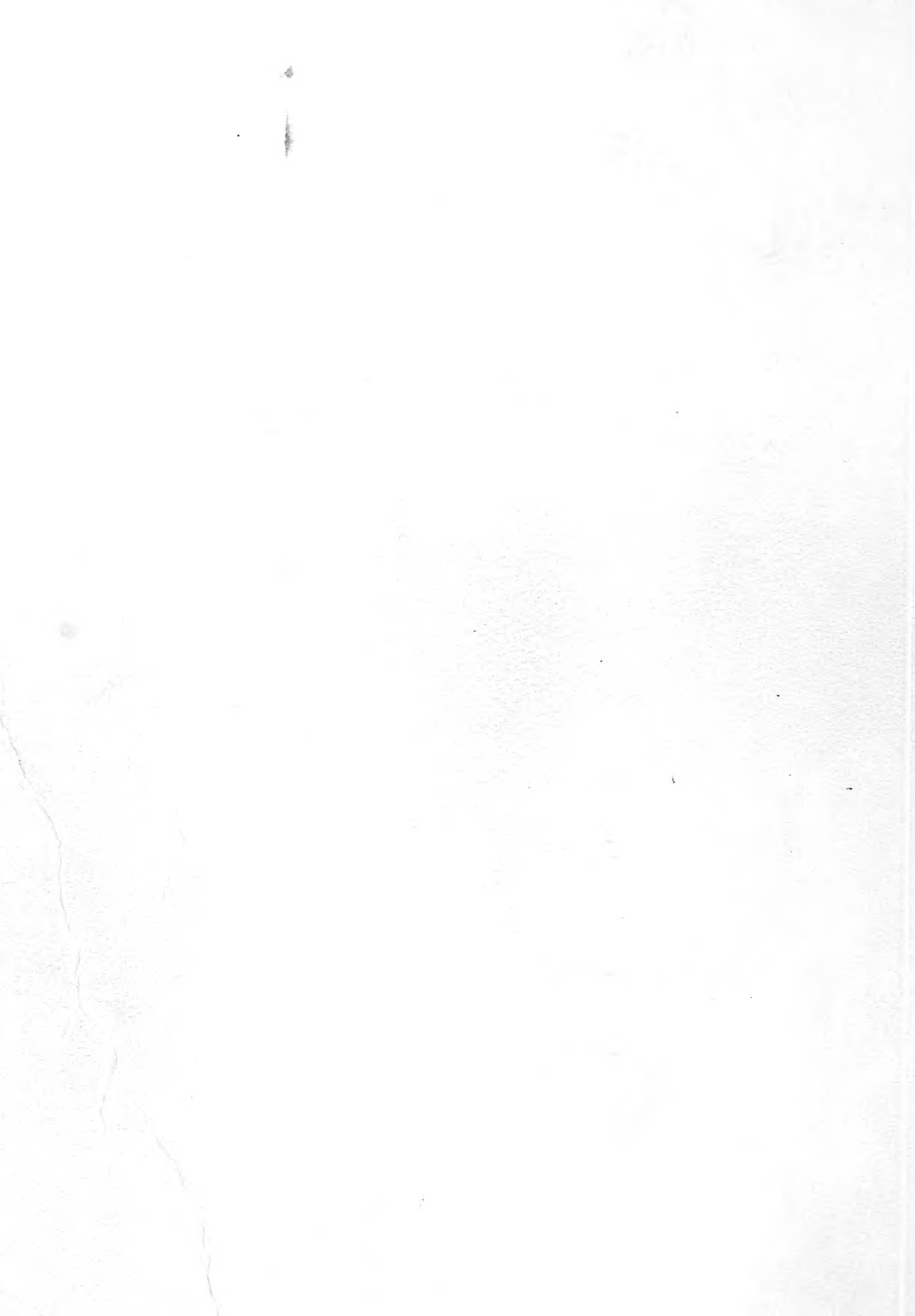


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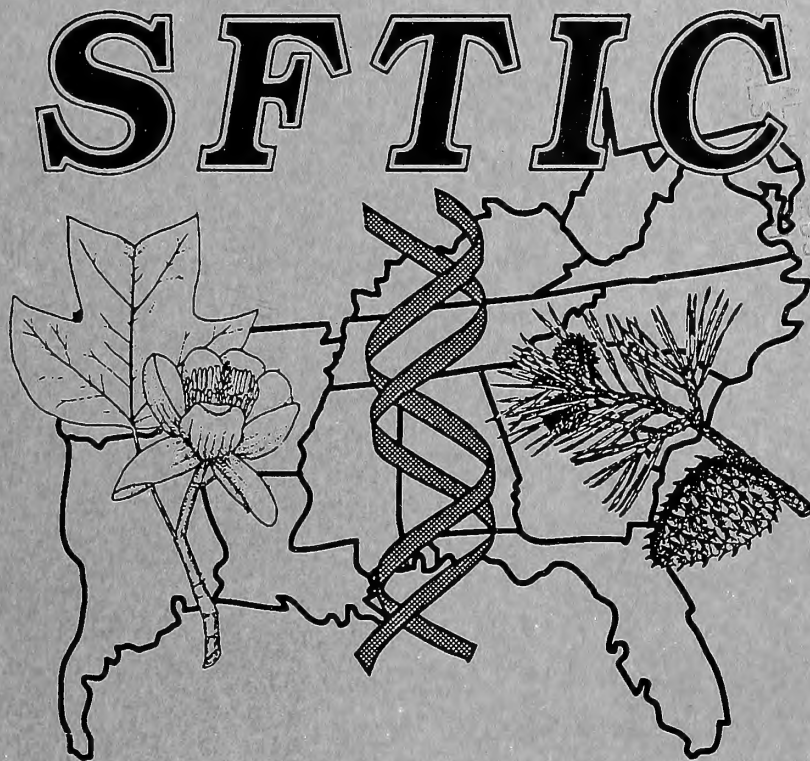
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PROCEEDINGS

22nd SOUTHERN FOREST TREE IMPROVEMENT CONFERENCE

June 14-17, 1993
Atlanta, Georgia



SPONSORED BY
THE SOUTHERN FOREST TREE IMPROVEMENT COMMITTEE
IN COOPERATION WITH
The Institute of Paper Science and Technology
Georgia Forestry Commission
USDA Forest Service-Southern Region
The University of Georgia Cooperative Extension Service

Proceedings of the 22nd Southern Forest Tree Improvement Conference
Compiled by Clark W. Lantz and David J. Moorhead

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ANNOUNCEMENT

SFTIC MEMBERS:

The U.S. Department of Agriculture published the Pollen Management Handbook (Agriculture Handbook 587) in 1981. That handbook, edited by Carlyle Franklin, supplied up-to-date information about the biology, phenology, collection, extraction, storage, and utilization of pollen and discussed the management of pollen to increase genetic gain. Publication of this information increased tree breeders' awareness of the significance and importance of pollen management. Availability of Agriculture Handbook 587 greatly stimulated interest in pollen management, and increased interest led to additional research.

In 1990, the Southern Regional Information Group (SRIEG) sponsored a pollen management conference that was a forum for the presentation of information obtained through research since 1981. Sixty-seven people attended the meeting, which was held in Macon, Ga., on July 18-19, 1990 and scientists from the United States, Canada and Sweden presented papers on a range of topics. These papers have been collected in a second publication, Advances in Pollen Management, USDA Agriculture Handbook No.698. It is to be hoped that this publication will be as effective as the Pollen Management Handbook was in promoting interest and research in pollen management related to the production of improved conifer seed.

If you would like a copy of Advances in Pollen Management, please return the form at the bottom of this page to:

David L. Bramlett
USDA - Forest Service
Rt. 1, Box 182A
Dry Branch, GA 31020

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**PROCEEDINGS OF THE
22nd SOUTHERN FOREST TREE
IMPROVEMENT CONFERENCE**

JUNE 14-17, 1993

Atlanta, Georgia

**Sponsored Publication No. 44 of the
Southern Forest Tree Improvement Committee**

FOREWARD

The 22nd Southern Forest Tree Improvement Conference was held at the Holiday Inn - Buckhead, Atlanta, GA. The conference was sponsored by the Southern Forest Tree Improvement Committee, in cooperation with the Institute of Paper Science and Technology, the Georgia Forestry Commission and the USDA Forest Service - Southern Region. There were 176 registrants representing 21 states and six foreign countries.

A total of 59 presentations, four invited and 55 involuntary, were given. The voluntary papers were evaluated by the Southern Forest Tree Improvement Committee for the \$200 Tony Squillace Award (best oral presentation/written paper). The paper Diagnosing low seed and cone yields from controlled pollinations of southern pines; by D.L. Bramlett was selected to receive the Squillace Award. Congratulations to Dave for an outstanding contribution!

Fifteen posters were exhibited during the conference. The Baruch Foundation award of \$100 for best poster was presented to Dario Grattapaglia and Ronald Sederoff. Their poster was entitled: QTL Mapping in Eucalyptus using Rseudo-Testcross RAPD maps, Half and Full-Sib Families. Congratulations to Dario and Ron for an outstanding poster!

22nd SFTIC Conference Committee:

James L. McConnell, Chair (USDA Forest Service - Retired)
Ronald J. Dinus - Institute of Paper Science and Technology
Timothy LaFarge - USDA Forest Service
Clark W. Lantz - USDA Forest Service
Russell Pohl - Georgia Forestry Commission
Gaines T. Tibbs - USDA Forest Service

DEDICATION

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PhD University of Wisconsin, Madison

Professor of Forestry, Virginia Polytechnic
Institute 1970-93

Chairman - Research and Graduate Studies,
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SFTIC Chairman 1980-82

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1923-1993

BS + graduate studies Royal Dutch College of Tropical Agriculture; Deventer,
Netherlands

Indonesia 1949-53 Established coffee, tea and rubber plantations

Ethiopia 1954-56 Established rice plantations

Florida Citrus Experiment Station 1957-59

University of Florida Cooperative Forest Genetics Research Program 1959-88

Steven Spurr Award 1988 (SAF)

THOMAS FRANKLIN SWOFFORD
1913-1991

BSF University of Washington
MF Yale School of Forestry

US Army (US + Phillipines) 1942-46

USDA Forest Service 1934-1940; 1946-1974
Regional Geneticist - Southern Region 1959-74

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GENERAL SESSION

Vision For The Future

A VISION FOR TREE IMPROVEMENT -- A PERSPECTIVE FROM THE POTOMAC

C. F. Bey^{1/}

Abstract--Development of tree improvement programs in the future will be influenced by national and international resource needs, current trends, and public values. Increased population pressures and public concerns for protecting the environment will be the major driving forces that set program direction. In general, tree improvement programs and research will be broadened to include new aspects of genetic resource management. The management and research programs will emphasize new technologies with public perceptions in mind. A talented, professional, diverse workforce, with a passion for solving problems, serving people, producing resources, and caring for the environment will be needed to meet the challenges of the future.

INTRODUCTION

Thank you for the invitation to participate in the 22nd Southern Forest Tree Improvement Conference. I am pleased to be here to share a few thoughts, hopefully pertinent to you as managers, specialists and researchers in the South. It's good to be in Atlanta and to see some familiar faces in the audience. This brings back pleasant memories of my past work in this region.

I like the meeting theme of looking to the future. Hopefully, as we all strive to define a desired future, we can learn from each other and work together to achieve our goals. I will begin with a discussion of forces for change and current trends in natural resource management, followed by my vision for tree improvement.

No matter what position, organization, or perspective we come from, I think it is important to keep in mind the interrelationships or linkages in all that we do. That is important at all operational levels. A New York City Commissioner said it this way, "The environment is no longer an environmental issue." He went on to explain how environmental issues are completely intertwined with the whole fabric of society. In a similar way, I see tree improvement intertwined with the political, social, economic, ethics and science issues of today. These major issues are critical and central to our quality of life and the sustainability and health of the planet Earth. As tree improvement professionals, we have a key role in addressing the science problems and in serving a special set of societal needs.

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USING DIFFERENT PERSPECTIVES

I am pleased to see that we are beginning this meeting with a look at the topic of tree improvement from different perspectives. I view examining topics from different perspectives as part of our continuing education responsibility. Malcolm S. Forbes said, "Education's purpose is to replace an empty mind with an open one." If we use that definition, then examining different perspectives should be a routine practice. Oliver Wendell Holmes, stated it a little differently: "The human mind, once stretched to a new idea, never goes back to its original dimensions." New perspectives, like new ideas, can be forces of change.

Joel Barker, in his book, Paradigms--The Business of Discovering the Future, describes three keys to successful participation in the twenty-first century. They are anticipation, innovation, and excellence. It is the anticipation and innovation keys that drives what I want to talk about today. It deals with assessing future customer needs and techniques for being responsive.

I expect that the four visions on tree improvement you hear this morning will be different, each with an appropriate rationale, and each with a different perspective. Perhaps our first task is to listen and try to understand each others' perspectives.

WHERE HAVE ALL THE CHANGES BEEN

To set the stage I would like to reflect briefly on what has taken place in forestry in America over the past century. For convenience, I would like to break the past century into three phases: (1) exploitation (up to 1930); (2) protection (1930-60); and (3) management (1960 to present). In phase 1, forests were viewed as obstacles to agriculture; as a primary source of material for fences, bridges, railroads and charcoal; and often disposed of by burning. Around 1930 we moved to an era of protection, phase 2. In those 30 or so "protection" years, we reduced wildfire burns by a factor of 10. We initiated forest management practices and restored a lot of the cut-over and burned-over lands, especially in the East. Timber production was considered to be the primary purpose of forest lands. In phase 3, roughly the past 30 years, we accepted forest land as the producer of many goods and services. The forest became the provider of many resources--timber, water, wildlife, fisheries, recreation, minerals, etc. It appears that we are now moving into a new era, where forest values and land stewardship are central themes. What the forest "is" and the condition of the forest gets equal consideration with what the forest produces. While only time will reveal how this theme plays out, it appears to be the beginning of a paradigm shift. The boundaries of the concept are not completely defined and the measure of success is unclear.

Along with the changes concerning the purpose and value of forests, management and research on forest lands became much more diverse and complex. Think for a minute about our historical approach to natural resource management. In the first half of this century, forestry professionals were educated and expected to know what was best for the land, best for the mankind, and how to balance the two. Sometime during the 1960-70's, it became appropriate and popular for society to speak out on environmental issues. There were lots of milestones in this time period, e.g. the first Earth Day, the Multiple-Use Sustained-Yield Act, the Wilderness Act, the Land and Water Conservation Fund, the Historic Preservation Act, the National Environmental Policy Act, the Endangered Species Act, and the National Forest Management Act -- to name just a few. We addressed other issues dealing with fish, wildlife, clean water, wild horses and burros, insecticides, rodenticides, fungicides, resource planning, clean air, etc. That public involvement movement has grown to a point where forest managers now seriously consider what land management practices the public will and won't accept. That public (individuals, interest groups, Congressional representatives, etc.) are not bashful about expressing opinions that go beyond State and National Forest boundaries.

Within our profession we have welcomed and promoted public involvement and participatory management. We have placed greater emphasis on human values, as well as land and resource protection and restoration. Listen to the titles of three books reviewed in the January 1993 Journal of Forestry. They are "Balancing Act: Environmental Issues in Forestry," "Global Imperative: Harmonizing Culture and Nature," "On Common Ground: Managing Human-Planet Relationships." Our profession was not involved in reading and reviewing those kinds of books 10 or 15 years ago. These three examples demonstrate the kinds of changes that are occurring.

Have there been changes in society? Of course. In fact, they have been the primary driving forces for the changes in natural resource management. We have more people. They are more concentrated in cities and they have a higher standard of living. We have developed into a luxury-laden American society that increasingly places higher and higher values on natural resource protection and restoration. That luxury and those values prompts conflicts and challenges. As a rule it's utilization versus preservation debates.

It is clear that our society and our approach to natural resource management has changed a lot during the last century and particularly over the last 30 years. We could debate whether the glass is half empty or half full, but I think that we have adjusted to the major changes fairly well. I suggest that one measure of successful adjustment is the fact that there has been a continued need by the customers for our services.

THE LARGER PERSPECTIVE

I expect that some of you would argue that we and society have gone too far in public involvement, that the professional can no longer do his/her job, that

work efficiency is greatly reduced, and that the protectionism movement is wildly out of control. Some others would likely argue that we have been far too slow in adjusting to change and that we are not listening to what the public wants us to do. Where do we draw the line for public involvement and participatory management involving decisions about how our natural resources are used, restored and protected?

Lets digress and take a world perspective peek on what is happening to the environment. I refer you to Our Common Future, A United Nations Commission on Environment and Development report on the status of the world. The Report is often quoted with regards to the topic of sustainable development--meeting the needs of the present without compromising the ability of future generations to meet their own need. Here is a statement from this 1983 report: "Our Common Future serves notice that the time has come for a marriage of economy and ecology, so that governments and their people can take responsibility not just for environmental damage, but for the politics that cause the damage. Some of these policies threaten the survival of the human race. They can be changed. But we must act now." This report is a serious request for us to look at environmental and development problems beyond local, state and national borders.

I refer you also to State of the World 1993, published by Worldwatch Institute, another international group concerned about getting a sense of the condition of the world's environment. Here are a couple summary statements for perspective: "Today environmental degradation is directly affecting national and global economic trends." "Soil erosion, thinning of the ozone shield, air pollution, and increased flooding caused by deforestation are all taking their toll. Rangelands and oceans are being pushed to their limits, and their productivity is beginning to fall." Here is another call to understand our problems within the international framework, the larger perspective.

How do Americans feel about the environmental issues? The Times-Mirror Magazine National Environmental Forum Survey, completed in March, 1992, reports that 30 percent of Americans think of themselves as "active environmentalists" and another 52 percent say they are "sympathetic" to environmental concerns. Nearly two-thirds think environmental laws and regulations have not gone far enough. When faced with a choice, two-thirds come down on the side of the environment rather than economic growth. Perhaps most surprising, Americans are split evenly on the assertion by the World Watch Institute that the 1990's is the last decade when humanity will have a chance to save the Earth from environmental catastrophe. Whether it is true or not that we are on the brink of environmental disaster, it is important that we as natural resource managers and researchers understand how the public perceives the issues.

Do ideas and statements that I have just presented have anything to do with tree improvement in the United States? I would say "yes." In a very real sense the issues that effect Americans shape our ideologies, our programs and

our priorities. Understanding these issues can be very important as we initiate and develop our tree improvement programs.

BROADSCALE TRENDS

Each of you can see many problems that need to be addressed. You know the management issues and the scientific questions. I assume that your agenda provides a sense of current priorities for the South. I see topics on the list that were not here 10 years ago, but are current today. Hopefully, some of your efforts are addressing topics that are oriented toward the issues of the future.

In general, the following trends seem to be the major drivers from which all pressures on natural resource use and protection originate:

- (1) Population increases and changing demographics will put more pressure on the use of all natural resources;
- (2) There will be increasing public pressure for protection and restoration of natural resources and the environment; and
- (3) Global concerns, involvement and cooperation will increase.

Within this larger context, what are the natural resource and forest management trends? I believe that we will see increased attention given to ecological understanding, longer rotations, larger landscapes, new uses and new values for the land and natural resources, and special emphasis given to the condition of the resources. Regarding the condition of the resources, there will be increased emphasis on forest health and sustainability. We will hear a lot about ecosystems and ecosystem sustainability. By ecosystem sustainability I mean, the ability of the system to maintain biological diversity, resiliency to stress, and a capacity for renewal over time, while providing a flow of products or benefits that meet current or future human needs.

The emphases described above are now embodied in the USDA Forest Service, National Forest System philosophy for forest and range management. That philosophy is referred to as Ecosystem Management. In the National Forest System there are additional components of Ecosystem Management that deal with the operational aspects of the philosophy. These include choosing a desired future condition, looking at past conditions, examining natural system options, public involvement, partnerships, and guidelines for clearcutting. Its a philosophy that includes the human and economic components, commodity production, and protection. The dilemma of balancing production and protection exists today, as before, but with an increasingly difficult challenge of balancing current production needs with the long-term desired future condition of the resource.

TRENDS AND IMPLICATIONS FOR FOREST MANAGERS AND RESEARCHERS

Before I get to a specific scenario--a vision for tree improvement in the Forest Service--I would like to paint the portrait of what I see happening in forestry and all natural resource management.

For forest managers, I see the assignments becoming increasingly complex. There will be a need for highly educated and skilled managers. The ability to fit together all the pieces of the resources will require someone with strong analytical and synthesizing abilities. Managers will need to be visionary, take a broad perspective, and be problem solvers. Natural resource education will be important, as will the ability to understand complex ecological processes. Is that asking too much for managers? I hope not. They will be making long-term decisions that impact the land, influence resource availability of the future and effect peoples lives.

There will be an increased need for specialists, who can assist the managers. Specialist of many types will be needed to understand the function and structure of the broader ecosystem. Specialists will be needed to understand scale relationships--how do we scale-up from our understanding of organisms, to stands, populations, watersheds, and landscapes. We will need a lot more than just wildlife, fisheries, soils and recreation resource specialists. How about microbiologists, population geneticists, insect ecologist, and modelers for starters. Although emphasis will need to be on the multidisciplinary approach, the need for specialists will increase.

Should there be any changes in how we view professional development in preparing for the future? I suggest that managers and specialists will need to stay in one location for longer periods of time--to better understand the problems and to observe how ecosystem processes unfold. Improved data bases, Geographic Information Systems, decision support systems and better ways to characterize risks will undoubtedly be available to display management options, but there will be no substitute for the intimate land/resource knowledge for understanding what is best for maintaining sustainability.

For researchers, I see a parallel track. We'll need a cadre of superb analytical people and synthesizers, who can bring together information from many independent studies. These researchers will need to be good at sorting out the priority research needs and in designing multidisciplinary studies that test some broad-scale hypotheses. I see these integrating type of studies as capstones after some of the specific, discipline-oriented research has been completed. This is an area that deserves special attention but should be approached with caution. Studies are likely to be large and consequently very expensive. Quality assurance for multidisciplinary type studies must be rigorous. If the questions asked and hypotheses being tested are mushy, so too will be the answers. Controls, replication, randomization and the level of uncertainty tolerable in the answer should be thoroughly addressed.

Managing research programs will not get any easier. Demands for answers to complex practical problems will likely increase. There will be requests and temptations to get into research on the latest problem-of-the month. In some cases, researchers will be asked to cooperate in demonstration projects and management trials. Its important that the researchers and managers make clear to each other their expectations of results for the projects being developed. The arena of quasi-scientific projects is fraught with dangers. Managers are prone to expect clear-cut and definitive answers to general questions. Researchers are prone to hedge on answers and want more specific testing. In these endeavors, managers and research need to "Beware."

There will be an increased desires to use science in establishing policies. Along these lines, some cautions are in order. While science should serve as the foundation for policy-making by establishing the expected results (options) and corresponding levels of risk, researchers must be alert to pressures for answers for research results with "correct" answers. It will be important to separate what is scientifically sound from what are research implications and expert opinions. While all may be useful in making management and policy decisions, and used as evidence in court, there are difference that should be made clear by researchers. I see an increasing need for quality assurance emphasis at all major research institutions. As researchers we need to be careful that we do not get in the policy-making game on the basis of science alone. Policy will generally deal with broad-based issues, where social, economic and political factors are also involved.

Research planning will increase in importance. Whether you are in industry, university, federal, or non-government organizations it will serve you well to have a documented plan for research. It should be strategic in the sense that it contains a vision, broad goals, and be long-term in nature. The plan should be coordinated and complementary with goals of other organizations. Such plans should change with time as problems are solved and new needs arise. The plans should be widely shared with other professionals. In general, I see too few of these plans. I suspect it is because there are too few.

Technology transfer will increase in importance. I'm a great believer in sharing what we learn in research with others. My early experience in Forest Service research at Carbondale, Illinois and working hand-in-hand with Burl Ashley in State and Private Forestry was an excellent example of how to get our walnut genetics and culture research implemented. There was an excellent feedback loop on current problems and everyone, especially the customer, benefited. I see a need for increased efforts in this type of technology transfer.

Within the context of increased partnerships and technology transfer, I believe that research must adhere to a certain level of independence. Rigorous adherence to scientific operational principles and the independence to present results, even if it is not consistent with current policy, must be maintained. To do otherwise will be to destroy research credibility. There will also be a need to express research results through risk assessment processes.

Natural resource managers and researchers of the future will need to give increased attention to education. Whether you call it continuing education or a life-long learning philosophy doesn't matter. Individuals without personal motivation, and/or a strong support organization will not serve society well. The society of the future will be different than it is today. Some in society will view the earth as an opportunity to explore and exploit the natural resources. Others will view the earth as the natural healer and provider that cares for us all. Most people will have views that fall inbetween. It will be our responsibility to have the expertise that presents the options to serve them all.

CURRENT TREE IMPROVEMENT AND GENETIC RESOURCE RESEARCH PROGRAMS IN THE FOREST SERVICE

With those ideas on the history, perspective and trends in mind, lets look at the tree improvement and genetic resource research programs that are currently developing in the Forest Service. Richard G. Miller, Forest Geneticist with USDA Forest Service has recently outlined the major goals for the Genetic Resource Program for the National Forest System. The plan developed in 1992 focuses on the next five to ten years and was crafted by National Forest System and Research geneticists, silviculturists, an ecologist, seed orchard managers, and representatives from two tree improvement cooperatives and a University. From the plan, the five major thrusts for the future are:

1. Provide leadership in developing and implementing gene conservation and genetic resource management programs.
2. Integrate genetic principles into the development, implementation, and monitoring of Forest Plans.
3. Maintain tree improvement programs to produce selected genetic materials and to provide genetic knowledge for appropriate tree species.
4. Improve understanding of genetic resource management both internally and externally.
5. Maintain, improve and expand linkages, partnerships and other cooperative efforts.

In general, the program aspects of this plan are considerably broader than in the past. The traditional tree improvement program still exists, but is does not dominate the activities as it once did. Part of this comes from the maturing status of seed orchards, the decreased need for seed for planting on National Forests, and the rising interest in genetics as a component of biodiversity and ecosystem management.

In a similar vein, we looked at the genetics research in the Forest Service, and made some projections for programs for the future. If we examine what has happened in the Forest Service over the past 10-15 years, we can see that research on the traditional provenance/progeny testing and breeding has decreased in several areas. There are few new studies along these lines, and a number of studies have even been put on hold for future measurements. In some cases, we are using those older studies for new purposes, e.g., looking at the genetic aspects of global climate change.

Overall, genetics research in the Forest Service has broadened over the past decade. There has been an expansion to now include genetic mapping, insect and disease genetics, genetics of stress resistance mechanisms, conservation biology genetics, and genetics of fungi in bioprocessing and bioremediation. The size of the program has decreased over the past decade, in about the same rate as has the total Forest Service Research budget.

A TREE IMPROVEMENT VISION

Historian Harold K. Steen said, "One of the many things we learn from history is that people at a given time often do not understand what is happening or what the real issues are." If I really knew the critical issues of today, I would feel more confident in my vision for tree improvement. Yet, I am not sure that my vision would be any more accurate.

For the near-term future, the next decade, there is a very realistic question of whether or not current trends will continue. And if they do continue, what might be the implications for tree improvement. For the next decade, I believe we will see increases in the genetic resource programs that have emerged and taken hold over the past several years. That would include genetic engineering, gene conservation, asexual propagation, genetics of stress resistance, and population genetics.

While these topics seem like the most likely winners for increases in the next decade, I don't see them increasing without some resistance. The idea of using genetic engineering to increase forest production, as in agriculture, will meet some opposition. Despite public sentiments, the pressures of interest groups and the national debt situation, I am convinced that genetic engineering for tree improvement will occupy an increasingly greater role. In food crops, within a few months we may see the first genetically engineered food crop, the tomato, on the shelves in stores. It will look and taste like other backyard tomatoes. The difference is an antisense genetic construct that reduces rotting, thus allowing it to be picked later in the ripening phase, stored longer and taste better than those picked green.

It appears that the introduction of the tomato into the market place may be just the beginning. Since 1987 there have been over 400 environmental release permits issued by USDA Animal and Plant Health Plant Inspection Service (APHIS) to field test genetically engineered plants. The experience in evaluating

these tests has led to the modification of the field testing approval process. In essence, there is now only a notification process for the introduction of six crops with which APHIS has had extensive experience (see Federal Register, Vol. 58, No. 60, March 31, 1993). Although there are no tree species on that list, there is now a petition process allowing for a determination that specific plant species may be put on the list. The way we will proceed with trees is being paved by experience with corn, cotton, potato, soybean, tobacco, and tomato testing programs. There are still numerous public issues to be resolved, especially in the labeling of food products.

It is important to recognize that there is considerable resistance to genetic engineering. During the next decade, I think it will be increasingly important to understand how the public perceives the science and practice of this growing discipline. Concomitantly, we will need to understand the paradigm that may be emerging. The current, production paradigm seems to be based on the idea that new breeding techniques, fertilizers, pesticides and improved cultural methods are useful, safe, and generally acceptable. The boundaries for the techniques are reasonably well recognized. Emphasis is on the improved product. To the researchers, managers and most of the public, the paradigm is rational.

When we move to genetic engineering, there are some new perceived dimensions to the paradigm. Perhaps most prominent is the notion that genetic engineering is tinkering with the integrity of nature, messing with the mystery and centrality of life, entering the sacred ground, altering the holy grail of DNA, etc. The limits of what might result from such tinkering are perceived as unclear. Its perceived as a pervasive endeavor, perhaps irreversible. To some it will be worse than the introduction of kudzu or multiflora rose. By most of our standards, this all seems irrational. Yet to be successful in genetic engineering we may have to recognize and perhaps operate in the paradigm of pervasiveness. That will require understanding public perceptions, getting serious about doing more social science, and restoring trust with the public.

Over the next decade, I see us gradually recognizing that our production paradigm is not totally appropriate. As we do, the tension between the two paradigms will be reduced and genetic engineering more readily accepted. My sense is that the situation will not be any worse than what already exists. Whether that is optimistic or pessimistic depends on your own perspective. It seems likely that other world-wide natural resource problems will become so critical that the tree improvement issues will appear to be diminished. Just two examples--I expect that water supply and water quality problems to get a lot worse over the next decade. Similarly I expect that we will hear a lot about forest health and how to restore ecosystems. Tree improvement will contribute to the later, but is not likely to be center stage. None of this diminishes our problems or our role, it just puts tree improvement in a different perspective.

I expect one of the things that we will accept in the next 10 years has to do with what some call the new world order of "self-organization." We will learn how tree improvement can reinforce the geo/bio/physical world (the

environmental system) to maintain or maximize the potential of the system. We will get away from the question of, "what is the impact?" to asking, "what can a project do to reinforce other things already in the system?" We will come to understand that "ecosystem sustainability" has more to do with how we adjust to living with the pulses of ecosystems than it is about reaching some steady state condition. This will develop as we come to realize the power of systems to self-organize. Tree improvement will be recognized as programs that primarily mimic the growth and decline cycles of natural forests.

In a similar fashion, in the next decade I see us emphasizing how tree improvement links to sustaining the ecosystems. We will be as concerned about the linkages regarding the function of tree improvement and other phenomena as we are with tree improvement processes per se. We will be concerned about linking things at different scales, i.e., molecular level to the ecosystem level. We will be concerned about linking genetics and tree improvement to forest health, biodiversity, sustainability, resiliency and other conditions of the forest resource. We will be concerned with linking the genetics of trees to genetics of other organisms. All this will require increased interactions with other disciplines and a broadened view of tree improvement.

CONCLUSION

Oprah Winfrey is quoted as saying, "when I look at the future, it's so bright it burns my eyes." I believe that the future for tree improvement and natural resource management and research will be bright. There will be problems, but we will solve them. It will take a plethora of talented and diverse men and women from the crosscut of society. We will need people who are inspired, with a passion for solving problems, serving people, producing resources, and caring for the environment. I hope that all of you are working to develop that cadre of caretakers, managers, specialists, and researchers.

I would like to close with the same notion that I began. Perhaps we can learn from the New York city commissioner who said "The environment is no longer an environmental issue." I say to you, genetic resource management and research is no longer a natural resource nor even an environmental issue. The issues are intertwined with the political, social, economic, ethics and science issues of today. These are major issues, critical and central to our quality of life and the sustainability and health of the planet Earth. The fact that the issues are bigger than us does not diminish our role, it only means we must adjust to a broader perspective. As genetic resource professionals, our role is key. I trust that we are all committed to adjusting to that broad perspective and to serving those special needs of society. That is indeed a noble charge.

THE FUTURE OF TREE IMPROVEMENT AS SEEN
FROM THE POINT OF VIEW OF THE STATES

by
J. P. van Buijtenen¹

ABSTRACT

This paper examines the future of tree improvement in light of major changes in the global economy as well as ecology. It also reports on the responses of state forestry organization to current stresses.

The world population is increasing by 250,000 people each day and is expected to double by the year 2050. The demand for wood will probably increase even more due to the increasing standard of living in many countries.

Multiple use forestry appears to be giving way to restricted use of forests. As a consequence wood production is more and more confined to private holdings. Much of the wood needed will need to be produced on relatively few acres. Genetic improvement will be an important tool in achieving this.

State concerns vary greatly from region to region. A shared concern of all state forestry agencies is the reduced funding available due to the many competing demands on limited state budgets.

Keywords: Global economy, sustainable development, resource management, restricted-use management.

INTRODUCTION

Public perception of forestry has radically changed over the last decade and in response forest policy at the national level has changed dramatically as well. What do these changes mean for

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forestry and how are they likely to affect tree improvement?

STATE OF THE WORLD

In today's global economy, one cannot look at the future of forestry and tree improvement without looking at the future of the world as a whole. Obviously the world faces some increasingly serious problems in the form of increasing CO₂ levels, increasing levels of pollution, global warming, and potential exhaustion of mineral sources of fuel. All these concerns are driven by the threatening overpopulation of the world. Currently the population of the world increases by 250,000 people every day. This is the equivalent of adding the entire US population to the world every three years. By the year 2050 the world population is expected to have doubled compared to now. In addition the standard of living is increasing in many developing countries. Together this will put an enormous strain on the world's resources.

A recent book (Rörsch and de Hart, 1993) discussed a number of potential scenarios for future development of the world. I will briefly quote two of them in this context. The pessimistic scenario essentially says that the human population is already exceeding the carrying capacity of the earth and that it is too late to prevent disaster. The optimistic scenario, on the other hand, says that there is a technological fix for every problem. The latter is highly questionable while the pessimistic scenario could possibly be true.

A key concept popularized by the United Nations report, "Our Common Future" (1992) highlights the concept of sustainable development. This is somewhat of an oxymoron since, taken literally, development means growth, and growth by definition is not sustainable. However individual processes can be sustainable. In other words forestry and agriculture could be sustainable given the right technology.

Another important point made in both books is that one should look at these problems in three dimensions: ecology, technology and economy. Most often one looks at many situations in only one or two dimensions which gives a very incomplete picture. I'll come back to this shortly with some concrete examples. In reality, many problems have more than three variables, so in effect we are dealing with a multidimensional problem.

Let's go back now and look at some specific examples in forestry of one-, two- and three-dimensional approaches. The Spotted Owl controversy on the west coast is largely a one-dimensional approach with overwhelming consideration of the ecology, but very little attention to any other factors. On the other hand the usual way we look at plantation management is in two dimensions, considering only the technological and economical aspects. My feeling is that if we took the ecological aspects into consideration many of those aspects would actually look quite favorable. For instance, tree plantations will restore degraded soils, reduce atmospheric CO₂ levels, and provide a habitat for wildlife. In some cases they would restore the native vegetation, in other cases they might replace it with a different vegetation. The latter could be considered a negative factor.

Another interesting article pertinent to our problem appeared in Science not too long ago (Ludwig et al, 1993). It was concerned with principles of effective resource management and gave a number of good fishery examples, and a forestry example that seemed a little off-base. However they had a number of rules for effective resource management that seemed quite sensible. They deserve to be quoted in full:

- "1. Include human motivation and responses as part of the system to be studied and managed. The shortsightedness and greed of humans underlie difficulties in management of resources, although the difficulties may manifest themselves as biological problems of the stock under exploitation.
2. Act before scientific consensus is achieved. We do not require any additional scientific studies before taking action to curb human activities that effect global warming, ozone depletion, pollution, and depletion of fossil fuels. Calls for additional research may be mere delaying tactics.
3. Rely on scientists to recognize problems, but not to remedy them. The judgment of scientists is often heavily influenced by their training in their respective disciplines, but the most important issues involving resources and the environment involve interactions whose understanding must involve many disciplines. Scientists and their judgments are subject to political pressure.
4. Distrust claims of sustainability. Because past resource exploitation has seldom been sustainable, any new plan that involves claims of sustainability should be suspect. One

should inquire how the difficulties that have been encountered in past resource exploitation are to be overcome. The work of the Brundland Commission suffers from continual references to sustainability that is to be achieved in an unspecified way. Recently some of the world's leading ecologists have claimed that the key to a sustainable biosphere is research on a long list of standard research topics in ecology. Such a claim that basic research will (in an unspecified way) lead to sustainable use of resources in the face of a growing human population may lead to a false complacency: instead of addressing the problems of population growth and excessive use of resources, we may avoid such difficult issues by spending money on basic ecological research.

5. Confront uncertainty. Once we free ourselves from the illusion that science or technology (if lavishly funded) can provide a solution to resource or conservation problems, appropriate action becomes possible. Effective policies are possible under conditions of uncertainty, but they must take uncertainty into account. There is a well-developed theory of decision-making under uncertainty. In the present context, theoretical niceties are not required. Most principles of decision-making under uncertainty are simply common sense. We must consider a variety of plausible hypotheses about the world; consider a variety of possible strategies; favor actions that are robust to uncertainties; hedge; favor actions that are informative; probe and experiment; monitor results; update assessments and modify policy accordingly; and favor actions that are reversible."

I would like to add a few rules that seem to be borne out by observation, which could be called Murphy's Laws of Resource Conservation:

1. If wood is desperately needed it will be cut regardless of future consequences. Looking at world history this seems to be the general outcome. I have little optimism that human nature has changed much recently. There is a corollary to this.
2. Environmentalism is a noble philosophy largely dependent on affluence. This can be framed in a different way which was

brought home to me personally² pretty strongly during World War Two:

3. If your main concern is to survive the next twenty-four hours the consequences for the next century are irrelevant.

Some of these considerations seem somewhat remote from my assigned topic, but I hope they provide a useful philosophical framework from which to view the following discussion.

THE FUTURE OF TREE IMPROVEMENT

Most efforts to predict the future are rather unsuccessful and I will therefore not attempt to do this. There is a more useful way of looking at it: the future doesn't just happen, collectively we make it happen. A good example is the organization of the SFTIC conference. We started planning this two years ago, and amazingly, it is happening on schedule as planned. Usually all goes well, except for an occasional mishap. In tree improvement we are doing very much the same thing. We are planning our seed orchards and progeny tests based on predicted planting programs and although changes beyond our control do take place, much of it actually develops as planned, although maybe a few years behind schedule. The question to ask therefore is what do we want to happen.

Let us accept as given that the philosophy of forest management is drastically changing. I grew up with the concept of multiple use management and as near as I can tell the concept is nearly dead. Instead we see a number of much more restricted uses. Examples of this are the use of forests as wilderness areas, national forests, parks, recreation areas, areas used for protecting endangered wildlife and finally production forestry which is taking place mostly on private lands, both industrial and nonindustrial. Accepting that this is not expected to change in the near future, we should make a vigorous case that production forestry is not only a legitimate land use, but is essential to the affordability of all the other uses.

Another thing that is of some concern is the need for crop rotation in forestry. Based on experience with other species such as Scots pine in the Netherlands and Christmas trees in the South,

² During the last year of World War Two, my family and I survived on sugar beets and tulip bulbs, and cut every tree in our yard to keep warm and cook.

one can expect to see productivity losses after about three rotations. Evidence is beginning to accumulate that this is also the case for loblolly pine. We need to start thinking about alternatives to loblolly pine. This could be hardwood species such as sweetgum and various oaks, or agriculture crops. Planting improved trees might be appropriate in some cases, in other cases the best alternative could be clearcutting the areas and letting the hardwoods already present on the site reproduce by sprouting.

The above pertains particularly to industrial and non-industrial private land holdings. The following section speaks more specifically to the views of the state agencies.

RESPONSES OF STATE AGENCIES

To complete my assignment I contacted most of the state agencies. Following is a summary of the main points made in these conversations.

One thing that was obvious after talking to several agencies was the tremendous difference between regions. For instance, the state of South Carolina was mostly concerned about the effects of Hurricane Hugo, while the state of Virginia was much more concerned with the increasing value of land as real estate, because the land owners are less and less interested in the production of timber on their land, but consider it rather as a place to put a second home. All of the states face very severe budget pressures, which over time could negatively affect tree improvement programs.

A number of state foresters made the point that in many areas planting is the only option. This particularly pertains to land that is currently not forested. Planting programs are heavily dependent on incentive programs. Planting on private nonindustrial lands reached a high of 1.4 million in 1987, leveled off to a low of about 750,000 acres in 1992 and seems to be increasing again in response to the high current stumpage prices.

Many of the state foresters made the point that, because of the greatly reduced area available for production forestry, more and more wood needs to be produced on fewer and fewer acres, primarily on private lands. As a result they need to be managed as intensively as possible and tree improvement will be very much needed in the future to insure high productivity, while maintaining or improving quality.

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FOREST INDUSTRY'S INCREASINGLY PROBLEMATIC JOURNEY INTO THE FUTURE

R.A. Woessner¹

Abstract. -- The southern wood products industry has been committed to establishment of the southern pines in plantations for over 70 years. Likewise, the industry has been applying genetic principles to improving the growth of the trees in these plantations for over 40 years. The goal, of course, was to produce fiber to be consumed in company mills. An investment in plantations lasting about a quarter of a century is justifiable if sustainability of supply is assured as well as the maintenance of a competitive advantage. Until recently, growing highly productive forests for harvest was looked upon favorably by the public. Now, the industry is increasingly berated for its cutting and intensive management practices. This includes the use of genetically improved planting stock.

The ever lengthening list of business and environmental issues facing executives in segments of the forest products industry make it increasingly difficult to choose a profitable path to the future with a reasonable level of risk and a competitive rate of return. This paper reviews a number of these interacting issues. The research community can buffer the risks by assuring that the genetic integrity of the industrial high yielding plantations is beyond question.

INTRODUCTION

The southern wood products industry has been committed to forest renewal for over 70 years. U.S. Forest Service records indicate a modest beginning as only 15 thousand acres were planted on industry land in 1925(U.S.F.S. 1988). Interestingly, this was 48 percent of the acreage planted that year. Plantation establishment on industry land in the South has now grown to over a million acres per year in the 1990's. The forest industry is now responsible for over 60 percent of the tree planting in the South. Industry plantations currently occupy over 16 million acres. The 55 percent of the land area of the 12 southern states that is forested totals 182 million acres. Thus, one acre of every eleven forested acres is an industry pine plantation. If Forest Service projections to 2030 come true, then one acre of every seven forested would be an industry pine

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plantation..

The industry has been committed for over 40 years to applying genetic principles to improving the trees used in establishing these plantations. These extremely successful programs are producing improved seed in record amounts. In excess of 60 tons of improved seed is being harvested in good years. Soon, 25 percent of the seed for plantation establishment will be from advanced generation orchards.

Until recently, growing highly productive forests for harvest and conversion to useful products was looked upon favorably by the public, who incidently are the very customers that buy the products produced from the forest. Now the industry is increasingly berated for the practices of clear cutting and the intensively managed pine plantations. Our increasingly urban and affluent public wants much more than wood from forests. This applies to public as well as privately owned timberlands. There is an increasing concern about the quality of life now and in the future as the U.S. and other developed countries move into what some term the "post-industrial society." Issues like biodiversity, forest sustainability, wetlands, air and water quality, endangered species, ancient and tropical forests, and ecosystem management swarm around practicing industrial foresters like a hive of killer bees. It was probably inevitable that these concerns be extended to the tree genetic program (Hoekstra 1992). The warning signs have been present for some time. Concerns have been frequently raised about the wisdom and safety of the genetic manipulation of agricultural plants and the medicines used to cure our ills. All these environmental issues are more likely to intensify before they diminish.

These environmental issues are an over burdening addition to the other current challenges facing the industry. Choosing a profitable path to the future with a reasonable level of risk and a competitive rate of return is the goal. The following section will review these interacting issues.

OVERVIEW

Wood, The Essential Raw Material

The growing of trees as crops to be harvested is big business in the South. Timber is the most important agricultural crop in the South. Wood ranks ahead of such other crops as soybeans, cotton, tobacco, wheat or corn (U.S.F.S. 1988). The portion of the industry most familiar to me, the integrated producers of solid wood products, pulp, paper and paperboard, maintain a competitive advantage because of the currently abundant and competitively priced wood supply in the South. Jaakko Poyry (1989) stated "that this preeminent position was in danger because of the diminishing supply of softwood pulp and neglected silviculture, particularly in the "U.S. woodbasket" of the Southern U.S." Recently, Colberg (1992) reported that softwood inventories were declining in many areas of the South.

Wood is the single most expensive item used in the manufacturing process by the forest products industries. The industry must cost effectively produce wood while concurrently meeting the other expectations that the public wants from the forest.

The Industry

Any discussion of the forest products industry must address diversity. Diversity exists in location, size, capital structure, land ownership, product lines, manufacturing processes and marketing strategies et al. This diversity means it is extremely difficult to put forth one industry position. There are similarities among the largest companies. The largest wood consuming industries are frequently integrated producers of pulp, a wide variety of paper products and several solid wood products. Increasingly, these businesses have a global focus. In fact, their long term profitability depends on selling to foreign markets. Also, many are divisions of larger companies that sell a wide variety of consumer products. The majority of the company revenue comes from selling the consumer products. The pulp and paper operations supply the other businesses but are not the reason the companies exist as a commercial entity. These consumer products are sold in very competitive markets that bring the customer and the company selling them into very close contact from a marketing and sales standpoint. The end result is that customer expectations can greatly influence not only what is produced, but how it is produced. This means customer expectations in Europe or the Far East as well as the United States can ultimately influence how we produce and manufacture wood products in the South. An example is Germany's "Green Dot" legislation. No longer are purchase specifications (for pulp) in Germany limited to those pulp properties required to satisfy its intended use. Now the pulp must meet strict environmental performance standards at the mill producing it, and sustainable management practices in the forests from which the fiber comes (Wrist 1992). Another section of the law deals with recycling which is the next topic.

Recycled Fiber

As a result of governmental and public expectations, the consumption of recycled fiber is growing more than twice as fast as overall fiber consumption. This trend is projected to continue to 1995 and beyond. Recovery rates were 24 percent in 1985, 29 percent in 1991 and are expected to rise to at least 40 percent in 1995. As a benchmark note, the Japanese are working hard to push an already high rate of 55 percent recycled fiber to 60 percent. The basic technology to do this already exists for some grades of paper. The challenge is to improve on the existing technology. These recent and essentially mandated rapid increases in recycle use have a negative impact on company profits. Recycled products require additional capital and manufacturing expertise but bring no more in the marketplace at this time. Use of recycled fiber does however keep the producer in the market. Capital spent on recycle capabilities is not available to be spent elsewhere such as in the forest. Also, high usage rates of recycled fiber can tend to weaken the corporate focus on the primary fiber source which is of course the forest.

A Capital Intensive Industry

These capital expenditures for recycle capacity come close to being the proverbial straw that broke the camel's back. These expenditures are an additional unwelcome burden added to the balance sheet of the paper industry which is already the most capital intensive industry in the United States and probably the world. During the last ten years, the paper industry has been twice as capital intensive as the average of all manufacturing (Storat 1993).

Environmental protection is a big part of this. Expenditures for water and air quality protection and solid waste disposal are consuming billions of dollars of industry capital. The National Council of the Paper Industry for Air and Stream Improvement reported spending estimated at 1.343 billion dollars in 1991. These investments in environmental protection represented about 19 percent of all industry capital spending in 1991. These expenditures of over a billion dollars a year for environmental protection have been occurring since 1989 and are expected to recur in 1992. Spending for environmental protection could claim about 20 percent of industry capital during the 1990's (Storat 1993). Capital properly invested allows the industry to remain globally competitive. However, the capital invested must generate sufficient revenue to cover the costs of obtaining the capital plus provide returns to investors. Also, as mentioned previously, capital spent for environmental protection is not currently available to invest in the forest. However, this huge manufacturing base must be supplied longterm with an abundant supply of fiber that allows the U.S. industry to remain cost competitive.

Wetlands

The debate over wetland classification, protection and management is another environmental issue which clouds the business picture for the Southern industry. The most contentious issues regarding silvicultural operations in wetlands are harvesting, intensive site preparation, and planting. Other points of hot debate are minor or major drainage during harvesting and regeneration (Cubbage and Flather 1993). Although there are 26 million acres of southern forested wetlands, only 6.5 million of the pine or pine-hardwood type are involved in the latest debates over intensive management. Cubbage and Flather (1993) point out that the 6.5 million acres is a modest 3.6 percent of the total forested area in the South, but they are a crucial part of the most productive lands in some regions. A million here, a million there and pretty soon you are talking real acreage. The forester trying to manage timberland for a reasonable profit continually asks - where will it all end?

Ecosystem Management

Ecosystem management is the proposed solution for maintaining all the forest values. Logan Norris (1993) is the principal author of a SAF task force report entitled "Sustaining Long-Term Forest Health and Productivity." The summary of the report contains 11 key findings. Two are particularly troubling to anyone managing timberlands for a profit. Item three states "Traditional sustained yield management, focusing primarily on the production of commodities, is proving insufficient to meet the long-term needs of our society for the broad mix of forest resources. Increased awareness of this shortcoming, coupled with new knowledge, is causing society to change the fundamental policies under which forests are managed." Item nine further states "Today's economic theories and market forces present a barrier to sustaining long-term forest productivity. The key difficulty is assigning values to items or conditions which are not in the market place." Under ecosystem management, logging plans and forest practices would be based on ecosystems rather than on individual tracts of land. A landowner wishing to cut a particular tract or carry out a particular silvicultural activity would be required to show that his plans were compatible with the

entire ecosystem. This implies the imposition of government regulation upon the entire forested landbase. Industrial plantations are established to provide cost competitive fiber to company mills on an as needed basis. Regulation of cutting and management practices weaken the incentive for intensively managing land by decreasing the potential for a profitable return.

The southern wood industry is heavily dependent on the NIPF owners. Ecosystem management will probably weaken their incentive for intensive management much more than the industry.

Wood Fiber as a Raw Material

Foresters tend to equate paper making with wood. This has been true only in recent times. The first paper was made by the Chinese in 105 A.D. Cai Lun, the inventor, used hemp rope, rags and fishing nets. Chinese have made paper from bamboo, rice and wheat straw, hibiscus, silk, rattan, jute and flax. These papers are very strong but very coarse.

Papermaking began in this country in Germantown, Pennsylvania in 1690. Rags were the principal furnish. A recent newspaper article spoke of using residue cloth from making bluejeans in paper making. Recent articles in the TAPPI magazine presented research on wheat straw, bagasse, kudzu, sunflower stalks, vine shoots, cotton stalks and kenaf. Horn, Wegner et.al. (1992) found that kenaf CTMP fiber could be used as a reinforcement pulp instead of expensive semi-bleached softwood kraft fiber. Kaldor (1992) predicts an inevitable shortage of wood fiber. He proposes the use of fast growing annual fibers such as Kenaf as an environmentally acceptable alternative which would help save the worlds forest resources. Currently, in the United States, non-wood fibers such as cotton, bagasse, hemp, abaca, and kenaf provide just .3 percent of the paper industry's fiber needs. (Slinn 1992)

Hardwood species have increasingly been utilized by the papermakers in the South. Hardwoods were initially used by some mills because they were cheaper than pine and readily available. In many parts of the South this is no longer true. Also, procuring hardwoods during wet conditions poses significant environmental problems. Other mills use hardwoods because of the properties they impart to the paper. Recycled pine can replace some portion of the hardwood fiber.

As wood prices rise, even the home builders consider alternatives. Wood's traditional competitors, such as steel and aluminum, are hoping to gain market share. Other less traditional building materials are also testing the market place. According to the National Association of Home Builders, a builder in Northern California is promoting the "rammed earth" technique. This system compacts moist soil, sand and cement, then reinforces it with steel to make walls. An Arizona couple is using "green" marketing to sell its houses which are made from straw bales that are stuccoed or plastered over and then reinforced with steel.

One can conclude that any fiber source, such as wood, is potentially replaceable if a economical supply is no longer available.

The West Coast Impact

Half of the standing softwood timber in the United States is owned and managed by government agencies. This timber is mainly in the Western United States. The controversy over old-growth and endangered species has drastically reduced the annual cut. Federal timber volume under contract has dropped from 41.2 BBF in 1983 to 10.8 BBF in 1992. This significant and ongoing curtailment of supply continues to decrease western lumber production. The 1992-1995 production is expected to drop another 10 percent beyond the 1991 levels.

Projections through 1997 by Clear Vision Associates indicate 40 percent of the North American softwood lumber production will be Canadian as it was in 1981. However, there is a significant shift from the West to the South. In 1981 the South produced one-third of the nation's softwood lumber. The 1997 projection has the South producing 47 percent of the softwood lumber, an increase of 14 percent. This increased demand on a diminishing supply pushes prices up dramatically. Southern pine stumpage prices rise by 1997 to 133 percent over 1992. Southern pine chip prices increase by 17 percent from 1992 through 1997.

These high prices and increased demand put a tremendous pressure on the timber resources of the South. The resultant increased cutting will intensify the concern over environmental protection and hasten development of restrictive practices. More restrictions on harvesting and forest management are inevitable as the environmental standards are tightened. California is the model we can ill afford to emulate. At present, California has the most stringent and comprehensive forestry regulations in the United States (Porter 1993). These regulations cover both timber management and harvesting. Porter (1993) quotes Bob Shaffer, Associate Professor of Industrial Forestry at Virginia Tech, as reporting that timber owners in California net 30 to 50 percent less for their timber than owners in non-regulated states.

TVA Decision on Chip Mills and Chip Transportation

The recent TVA decision to deny barge terminal building permits for transporting chips on the Tennessee River to Boise Cascade, Donghae Pulp and Parker Towing is self-declared regulation of forestry practices. The major reason cited for denying the permits was the lack of environmental protection. The TVA said that the voluntary nature of the harvesting guidelines and the inability of landowners and forest interests to accept more extensive, special harvesting procedures was the reason for denial of the permits. The TVA was no doubt influenced by the U.S. Fish and Wildlife Service claim that 16-17 species would be jeopardized without extreme environmental regulation of land management practices and harvesting practices beyond those expected to be voluntarily practiced. The timberland owners in the 42 county area impacted by this decision clearly have seen the value of their timber resource decrease. The wood using industries can and will seek their fiber supply elsewhere. The APA reports that Stone Container Corporation is growing hardwood in Costa Rica because its assessment of the preservationist impact in the United States makes long-term domestic fiber supplies uncertain at best. Stone also is entering into contracts for cutting Caribbean pine in Venezuela.

Growth rates are several times greater and rotation lengths much shorter in Brazil and other South American countries. Our market is so attractive to them that they will comply with the "green dot" laws simply to sell their products.

Endangered Species, The Everglades and Other Ecosystems

The South has had its share of problems with the Endangered Species Act (ESA) and will see more. The controversy over the Red-Cockaded Woodpecker is southwide and ongoing. Recently, U.S. District Court Judge Robert Parker issued a preliminary injunction barring even-aged logging on the national forests in Texas. The charges against three civilian foresters at Ft. Benning, Georgia who had been charged with violating the ESA were finally dropped. This action began with a letter from the Sierra Club Legal Defense Fund threatening to sue the U.S. Army and the U.S. Fish and Wildlife Service. Several pages could be written about the times ESA issues have impacted the southern wood using industries.

However, forests are by far not the only area where there is public concern about environmental quality. Water quality and ecosystem degradation are big issues in the Florida Everglades. Farming, housing and man-made disruptions in freshwater flow are said to be causing significant modifications in the Everglades ecosystem. These changes in turn are threatening the commercial fishing industries in southern Florida for lobster, stone crab and shrimp. There is also concern about damage to the coral reefs. There is no cheap easy solution. Correction of the problems could boost the price of sugar and vegetables grown near the Everglades. Southern Florida's 5 million residents could be facing tough water-use restrictions and higher taxes to pay for water purifying efforts. Resolution of the problems will require ecosystem management on a broad scale. A similar scenerio exists for the Chesapeake Bay. The bay is impacted by a watershed 25 times as large as the bay itself.

Salmon in the Columbia basin of the states of Washington and Oregon is another example. The interacting forces of dams, irrigation canals, flood control projects, mines, crop lands, communities that have replaced wetlands, industrial parks and forest practices have all modified the environment so that salmon are negatively affected. The summation of all the changes in the Pacific Northwest are just now beginning to be understood according to Dr. Victor Kaczynski, a leading limnologist. Dr. Kaczynski states "Every industry, including the timber industry, is going to have to give up something for salmon. There are also government policies that must be changed, because these, too, are hurting salmon."

The message I get loud and clear from this type of information is that sooner or later forest practices in the South will be subject to more and more restrictions. The costs of environmental protection will move from the mills to the woods.

Forest Stewardship Council

An international body called the "Forest Stewardship Council" was formed in 1992 (Hill, 1993). The stated goal is to "set a worldwide standard for good forest management by promoting widely recognized and respected principles of good forest management." Organizations that become certified by the FSC have to

demonstrate accurate, verifiable procedures for tracing products from their source to the marketplace. The FSC has set forth 10 principles. Portions of these 10 that are particularly relevant here are as follows: "Forest management must minimize adverse environmental impact in terms of wildlife, biodiversity, water resources, soils and non-timber and timber resources." "Forest plantations should not replace natural forests; they should augment, complement, and reduce pressures on existing natural forests." This council was formed in response to the "green" movement. The points made are another version of the German "green dot law." This is a direct approach for environmental protection through the consumer. The premise being that more and more consumers are "demanding that forest management be environmentally appropriate, socially beneficial, and economically viable" (Hill, 1993).

SUMMARY AND CONCLUSIONS

What vision of the future can be drawn from all these interacting issues? What will happen in a general sense appears clear to me. The why is also clear. When and to what degree are partially up to all of us.

The world is a finite resource. However, our demands upon the world resource base continually increase. The general expectation is that "we" can and must do a more environmentally friendly job of satisfying these demands in the future than we have in the past. Unquestionably, the growing, harvesting, and processing of wood into products useful to society can be done in an environmentally acceptable manner. The southern forest products industry will continue to be a big player in this effort. The industry has already shown a willingness to spend capital dollars to make the manufacturing processes environmentally acceptable. A similar willingness will continue at the forest level. The industry will do whatever is necessary as long as these efforts allow the industry to remain globally competitive. However, no matter what we do, the criticisms will continue and the environmental wish lists will continue to grow. Public opinion, local, national and international will encourage increased government regulation of the management and harvesting of forests.

The integrated global forest product companies will find solutions that are acceptable to the public in order to maintain market share. One part of the solution will be very highly intensively managed plantations on selected sites. This intensive management will have a minimal environmental impact on the functioning of the ecosystem as a unit. Genetic improvement will be an essential contributor to the outstanding growth in these plantations. The genetic pedigree of these forests, just as much as their management, will be under scrutiny from the critics. The wise thing would be to expect this intensive scrutiny and make plans to exploit it to an advantage.

My intent here has not been to imply that we are faced with imminent change more drastic than we have been contending with on a steady basis. However, planning to meet future expectations in tree improvement requires a longer time span than in most other disciplines. Now is the time to prepare for the future.

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FOREST GENETICS IN A CHANGING WORLD A GENETICIST'S VISION OF THE FUTURE¹

L. Fins²

Abstract - The beginning of this decade has been marked by major shifts in public attitudes toward forestry and whole new belief systems about forests. Ecosystem management and biodiversity have become the new buzzwords. Multi-storied management with a reliance on natural regeneration is hailed as "kinder and gentler" forestry, while "clearcut and plant" is seen as environmentally destructive and technologically primitive. Diversity is touted as necessary for ecosystem stability, while the planting of genetically improved trees is assumed to result in impoverished monocultures on the brink of disaster. In response, many public agencies and some privately held corporations have begun to move away from the forest practices that traditionally utilize genetically improved trees and some tree improvement programs are currently in a precarious position. These decisions appear to be based on a set of beliefs and assumptions that are unsupported by the evidence. Nonetheless, there is some hope. There are tremendous opportunities for forest geneticists in the future. Our expertise will continue to be important to descriptive and restoration ecologists, physiologists, pathologists, and ecosystem managers, as well as to those who are still in the business of growing wood and wood fiber as a crop.

Keywords: Forest genetics, genetic diversity, ecosystem management, tree improvement, forest health.

INTRODUCTION

As the direction of forestry has changed over the last few years, and more and more public land is withdrawn from production forestry, many forest geneticists and tree breeders have become concerned that the decisions are short-sighted with limited attention to the long-range genetic implications of the new policies. Nonetheless, I believe there are tremendous opportunities for geneticists to make a significant contribution to the future of forestry, if we are willing to see and understand the realities of peoples' perceptions, work to change some of those

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perceptions and accept others, and perhaps most importantly, to commit our expertise to revitalize the profession in this country and around the world.

Clearly, there have been major shifts in public attitudes toward forests and forestry over the last decade. Ecosystem management, forest health and biodiversity have become the new buzzwords. Natural regeneration is hailed as "kinder and gentler" forestry, while "clearcut and plant" is seen as environmentally destructive and technologically primitive. Ecosystem diversity is touted as necessary for ecosystem stability, and planting genetically improved trees is assumed to result in genetically impoverished monocultures on the brink of disaster. In response, many public agencies and some private corporations have begun to shun the forest practices that have traditionally relied on genetically improved planting stock. They now emphasize functional diversity with little attention to forest productivity.

PERCEPTIONS AND MISPERCEPTIONS

These shifts in attitudes appear to be based on beliefs and assumptions (I have identified five of them) that are unsupported by the evidence and which have no basis in reality. The first belief is that ecosystems are inherently stable if people would simply leave them alone. The second belief is that diversity and stability are closely linked. Third is the belief that evolution has finely tuned ecosystems, with genotypes perfectly matched to their sites of origin and therefore all genetic diversity is important and should be preserved. Fourth is the belief that any manipulation of the forest results in a severe loss of diversity, and that tree breeding programs produce genetically depauperate monocultures that are somehow unattractive and at high risk. And finally, linked with these ideas, is the belief that "natural" is inherently "best".

First of all, there is a broadening body of information from fossil, pollen and midden records that indicates that ecosystems are not stable. These findings have been reviewed by Brubaker (1988, 1991) and by Betancourt et al. (1990) and it appears that even some of the important plant associations that we observe today did not exist on the landscape as short as 3,000 years ago. The conclusion one must come to is that ecosystems are indeed dynamic; and therefore stability is not an inherent characteristic of ecosystems.

To the second point, the stability/diversity hypothesis was shown to be false almost 20 years ago. Stability is not linked to diversity. This notion is unsupported by any evidence and is no longer accepted by the community of ecologists. Goodman (1975) reviewed the theory, the models and the evidence for this hypothesis in a thorough work and concluded that

"The expectations of the diversity-stability hypothesis are borne out neither by experiment, by observation, nor by models...Clearly the belief that more diverse communities are more stable is without support."

On the third point, while many studies have shown that forest trees are highly variable, few people understand that only a small fraction of that variation is distributed between populations (Hamrick et al. 1992) and local diversity may be as much a function of

"evolutionary footprints" as adaptation to the local environment (Strauss et al. 1992). Furthermore, we find that similar adaptive responses are repeated across landscapes, even landscapes as highly variable as the northern Rocky Mountains (Rehfeldt 1991). The deployment strategies used with loblolly pine show that relatively restricted seed sources can be well-adapted to the climatic and soil conditions across a much broader geographic area (van Buijtenen 1992). To sum up, it is quite clear that evolution has not at all finely tuned the ecosystem. Genotypes are not uniquely matched and nor are they necessarily even closely matched to the environments in which we find them. Rather, many genotypes appear to be physiologically buffered so that they can thrive over a broad geographic area.

To the fourth point, there still seem to be many questions as to whether or not variation is being maintained when harvest regeneration methods, thinning treatments or selective breeding are used. Several studies have been conducted to address this issue. As just one example, in 1982 we sampled phenotypically superior, sub-dominant and randomly selected Douglas-fir trees in each of two even-aged naturally regenerated stands in northern Idaho (Fins, unpublished). The stands were approximately 50 and 56 years old at the time of sampling. The samples were compared for levels of genetic diversity using isozymes. Our hypothesis was that there would be differences in allele frequencies among the groups and we suspected that the select trees would have slightly higher levels of genetic diversity than the randomly chosen trees, and that the subdominants would have generally lower levels of diversity than the first two groups. Interestingly, differences in diversity among the three groups were generally small and not consistent between stands. The phenotypically superior trees had the highest level of genetic diversity in one stand, the sub-dominant trees had the highest level in the second stand and the randomly chosen trees had the lowest diversity in both stands. While this was an admittedly small study, it does suggest that **among the leave-trees**, overall genetic diversity for non-selected traits is not necessarily reduced nor enhanced by selection on phenotypic characteristics.

Nearly all of the studies conducted thus far indicate that silvicultural treatments do not decrease genetic diversity in the next generation (Neale 1985; Shimizu and Adams 1993; Yazdani et al. 1985). One study showed that diversity was re-distributed somewhat with overstory removal in a multi-aged stand (Millar et al. 1991); another showed that genetic diversity increased in seed-tree stands compared to the controls (Woods and Blake 1981).

The question of diversity has also been addressed for genetically improved populations. On the phenotypic level, a study by James (1979) showed that radiata pine, even from a seed orchard, is still highly variable phenotypically. As a consequence, James ultimately recommended planting 4 times the final stocking rate in order increase the frequency of the phenotypically better trees in the final harvest stand.

Several studies have also investigated the levels of genetic diversity in biochemical traits in seed orchard stock as compared to natural stands. While one study of **loblolly pine seed orchards** (Hamrick 1991) showed a decrease in genetic diversity of seed orchards compared to natural stands, several other studies with Douglas-fir (reviewed by Carlson and Yanchuk 1990), and Sitka spruce and redcedar (El-Kassaby 1992) showed nearly identical diversity or increased diversity of the seed orchards over the natural stands.

Overall, the studies to date indicate that harvest regeneration does not decrease diversity; thinning does not decrease diversity, and current breeding programs do not necessarily decrease diversity and may even increase it relative to natural stands.

As to the fifth point, whether natural is "best" is primarily a value-judgment. However, we must remember that "natural" does not necessarily mean most productive, best adapted or most diverse. But, knowledge of the current "natural" condition is probably a useful landmark to monitor the direction of change, whether it be the inevitable change through natural selection or the deliberate change through selective breeding programs.

THE FUTURE

As for the future, ultimately, geneticists must be a part of the planning process, working with managers to **develop long-range strategies** for public lands. Such strategies must satisfy the public's desire for "natural" forests while maintaining high productivity. I favor a national policy that institutes forest zoning on public lands such that highly productive lands would be designated for production of wood and wood products (while maintaining good stewardship of the soil and water); other areas would be designated primarily for alternative uses including archive (conservation) populations as reservoirs for genetic diversity. We must understand and embrace the idea of a separation of the archive populations from the production and breeding populations (Savolainen and Kärkkäinen 1992). We must recognize that each type of population has a different function and likely different locations.

Furthermore, we should consider a division of labor, whereby a primary mission of the public agencies is to protect the archive populations on public lands while the private sector focuses on production of wood and wood products. Within the production populations, the focus must be directed to achieving gains. We must be willing to manage genetic diversity wisely, which may involve decreasing genetic diversity on a local level while maintaining it on a landscape level. This type of change would include some risk, as does any crop breeding program, diversity would be maintained and available as needed from the archive populations and additional (and perhaps new) diversity would be available from the breeding populations.

If we accept this division of populations, lands and labor, we must also help to allay the public's (and profession's) concerns that we are somehow destroying gene pools. We must publicize our efforts to preserve and utilize diversity in our natural populations, while emphasizing the need and demand for wood and wood products. We must help people see the parallel between raising trees and raising food and fiber crops.

We must help to **educate** the public, including children, public school teachers, the media, and members of our own profession about the ecological trade-offs of using metal and other non-renewable resources in place of the renewable resource of wood. We must all begin to give talks at local clubs, schools, talk to the press and so on. We must carry the message and help people make the intellectual connections. Forest ecosystems are dynamic and ever-changing. No amount of set-asides will keep them as they are today or get them back to how

they were yesterday. Diversity is not linked to stability. Genotypes are not necessarily tightly adapted to environments. New mutations come and go in the natural world; it is futile to try to preserve all variants. Breeding can **increase** overall levels of genetic variation. We must help people get past the idea that "natural" is permanent, is "best" and is somehow "sacred".

We must align ourselves with ecologists, physiologists, silviculturists, and growth and yield scientists. We can also work with the restoration biologists to ensure that they use appropriate seed sources in their work. We have much to contribute to research on the effects of global climate change and we should work to accurately characterize populations based on their ranges of adaptive behavior. Studies of forest health must include investigations of physiological genetics of adaptive and growth traits. An understanding of genetic variation in traits such as nutrient efficiency, drought resistance, length of the growth period, and cold hardiness will be particularly important. We must continue to research and monitor changes in genetic diversity in our forest tree populations. We must help evaluate the changes in productivity as alternative management strategies affect genetic structures and diversity. If inherent productivity is lost or gained as a result of genetic changes in populations, we must carry that message to the public and the profession.

So, ultimately, my message is that with the current losses of forest lands to other uses, it is time for us to redouble our efforts to make people aware of our ability to contribute to all sectors of forestry from basic research in forest health to ecosystem management to timber production. We must do everything we can to achieve maximum timber production on our best sites to produce the wood and wood products that the public demands. This approach could include selecting only the very best genotypes for production, even at the risk of narrowing the genetic base in the production populations compared to natural stands. A carefully crafted breeding program that rapidly generates new and varied genotypes to replace old ones will be critical to the success of this approach.

As I see it, tree breeding can and should follow directly in the footsteps of American agriculture. Rather than lament the current state of our profession, we geneticists should embrace this time as an opportunity to utilize our talents and knowledge more fully and to contribute to all aspects of the profession. We can become the most efficient producers of timber in the world and still be a model of responsible stewardship.

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SESSION 1

Seed Orchard Management

DIAGNOSING LOW SEED AND CONE YIELDS
FROM CONTROLLED POLLINATIONS OF SOUTHERN PINES

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Abstract.--Rapid improvement of southern pines requires efficient and effective controlled pollinations, but many controlled crosses produce few cones and low yields of filled seeds per cone. Using the known causes of seed failure and cone mortality, a method for relating these causes to problems with controlled pollinations has been developed. This paper describes diagnostic observations of seed failure and cone mortality and procedures to improve the seed yields from controlled pollinations.

Keywords: Tree breeding, pollen viability, pollen storage, Pinus taeda L.

INTRODUCTION

Controlled pollinations are used by southern pine tree breeders to produce seeds for progeny testing. Success with controlled pollination requires experience and knowledge of the reproductive process. Techniques and equipment vary among tree breeders, but the basic methods are similar for loblolly pine (Pinus taeda L.), slash pine (P. elliottii Engelm.), shortleaf pine (P. echinata Mill.) and Virginia pine (P. virginiana Mill.). The objective is to produce adequate quantities of filled seed so that progeny tests can include equal numbers of seedlings from each mating. Timely selection of the advance generations, therefore, depends on efficient controlled pollinations.

DIAGNOSTIC OBSERVATIONS

In controlled pollinations, low-quality pollen, poor timing, and poor bag installation can cause seed or cone losses but so can insects or fungi. Pinpointing the causes of losses requires careful observations. In the following discussion, the necessary diagnostic observations are described and the probable causes related to the controlled pollination process are discussed. For a general reference on controlled pollination and pollen extraction, storage and testing see "Pollen Management Handbook" (Franklin (ed), 1981) and "Advances in Pollen Management" (Bramlett et al. (eds) 1993). Seed and cone losses from insects are described in "Seed and Cone Insects of Southern Pines" (Ebel et al. 1975).

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First-year aborted ovules

Large numbers of ovules often are aborted during the first year of development in cones that have been control pollinated (Table 1). They appear as small remnants of the ovule rather than developed seeds (Bramlett et al. 1977). One cause is the lack of at least one viable pollen grain in the pollen chamber of the ovule. When ovules in control-pollinated flowers do not have adequate pollen, the most likely reason is that pollen was not applied at the time of peak female receptivity. Open-pollinated flowers are exposed to wind-born pollen for extended periods, but the tree breeder applies pollen for only a few seconds. It is thus imperative that the pollen reaches the micropyle during the pollination process. In a timing study on loblolly pine flowers, Bramlett and Matthews (1983) reported that the average number of pollen grains per ovule was not significantly different from wind-pollinated flowers when the control pollinations were completed within 2 days of maximum flower receptivity (stage 5) or within a 4-day window with flower stages from

Table 1.--Diagnostic observations, probable causes and procedures that may reduce losses of ovules and seeds from controlled pollinations.

<u>Diagnostic observation</u>	<u>:</u>	<u>Probable causes</u>	<u>:</u>	<u>Procedures to reduce losses</u>
A. First-year aborted ovules (> 20%)		1. Inadequate pollination	.	Check timing of pollen application Check amount of pollen applied Check pollinator for distribution in bag Avoid pollinating wet bags
		2. Low pollen viability	.	Complete <u>in vitro</u> germination test Check collection, extraction and storage procedures
		3. Insect damage	.	Check pest management program for seedbug protection
B. Second-year aborted ovules (> 10%)		1. Insect damage	.	Check pest management program for seedbug protection
		2. Developmental Problem	.	No known treatment. Possibly related to water stress (clonal)
C. Empty seeds (> 25%) per cone or 15% of total seed)		1. Low pollen vigor	.	Check pollen extraction and storage procedures.
		2. Embryonic lethal alleles	.	Check parents for relatedness
		3. Seedbug damage	.	Check pest management program
		4. Fungal damage	.	No known treatment (clonal)

4.5 to 5.5. Control pollinations that were earlier or later than this period had statistically less pollen per ovule and an increasing percentage of unpollinated ovules per cone.

When there is a wide range of developmental stages of the individual flowers within a given bag, multiple pollinations of that bag are needed. For example, if flower stages range from 3.0 to 5.5, a second or even a third visit are needed to get adequate pollen into each flower. Multiple pollinations, however, are not efficient or beneficial if flowers are only two days apart in receptivity stage.

Distribution of pollen to the individual ovules is the function of the pollinator. The cyclone pollinator distributes pollen within the bag very effectively (Matthews and Bramlett 1981), but other pollinators, including a syringe and needle, a plastic wash bottle, and a camel's hair brush, also can be effective. From my experience, the type of pollinator is not as important as the correct timing of the pollination. Any of several available pollinators can give good results. Tree breeders should avoid pollinating bags that have water droplets condensed on the inside of the pollination bag.

For continued ovule development, at least one pollen grain must germinate in the ovule and begin growth through the nucellus tissue. If pollen does not germinate, the ovule aborts. Matthews and Bramlett (1986) looked at the effect of the percent viability of pollen on the eventual seed set. The development of filled seed is a function of the average number of pollen grains per ovule. The pine pollen chamber can hold up to 7 pollen grains. A normal seed will develop if just one of these grains germinates properly. Thus, with good pollen distribution per ovule, serious reductions in the number of ovules with viable pollen do not occur until pollen viability is 50% or lower. Even pollen with relatively low viability can produce reasonable filled seed yields. However, for high seed yields, tree breeders should use pollen with high viability.

To compensate for low pollen viability, the number of flowers pollinated should be increased rather than increasing the amount of pollen applied per bag (Bramlett et al. 1985). Filled seed yields from controlled pollinations can be increased by increasing the quantity of pollen applied per bag, but adequate seed set can be achieved with relatively low pollen amounts per bag (Bramlett 1977, Matthews and Bramlett 1986). If the pollen supply is plentiful I recommend applying 1.00 cc of pollen per bag with the cyclone pollinator (Matthews and Bramlett 1981). If the pollen supply is limited, the cyclone pollinator can be successfully used to deliver as little as 0.25 cc per bag. If less than 0.25 cc of pollen are available per pollination, a camel's hair brush should be used.

The third cause of first-year aborted ovules is the feeding of the seedbug Leptoglossus corculus (Say). Second-stage nymphs of this insect feed on southern pine conelets and penetrate the ovules. This damage halts ovule development and the net result is an aborted ovule that is indistinguishable from one caused by a lack of viable pollen. Insect damage can be reduced by a reliable pest management program.

It is important to distinguish insect damage from pollination problems. To estimate the number of first-year aborted ovules due to other causes, wind-pollinated cones can be collected and the aborted ovules counted. If the wind-pollinated cones also have large numbers of first-year aborted ovules, then it is a safe bet that insects are a major cause of the damage.

Second-year aborted ovules

As far as we know, ovule abortion during the second year of development is not related to pollination (table 1). Seedbugs are the only confirmed cause. These ovules begin enlargement in the second year but abort before the seedcoat is fully developed. Typical second-year aborted ovules are flattened or sunkened. They may be as large in outline as a mature seed. They frequently are filled with resin that exudes from the damaged ovule. If seed orchards have an adequate pest management program, second-year aborted ovules are rare.

Small hardened second-year ovules are sometimes found in mature cones. The cause of this seed loss has not been determined, but in most cases it accounts for less than 1% of the total seed potential. Occasionally, certain clones will have a large number of these second-year aborted ovules but the cause and cure are unknown.

Empty seeds

When the number of empty seeds in control pollinated cones exceeds 25 per cone or the proportion is greater than 15%, the loss is large enough to indicate problems that could be corrected. Unfortunately there are at least four causes of empty seeds in pines (table 1). Insects are the major cause of empty seeds in unprotected orchards or in natural populations of pines. The damage is caused by feeding of the seedbugs, L. corculus and Tetyra bipunctata (H.-S.) and by seed worms (Laspeyresia spp.). Adequate protection of seed orchards is currently provided by insecticide applications. Breeding orchards should also be protected at the same level to prevent losses of control-pollinated seeds. Small breeding orchards, especially those without buffers, may require adjacent areas to be treated to insure adequate protection.

Fungi are also known to cause empty seeds in pines, but no preventative fungicide applications are available to reduce the losses. Fortunately, the average loss to fungi is low, but losses in some individual clones may be substantial.

Embryonic lethal alleles can cause empty seeds. On the average, an individual pine has 10-12 recessive embryonic lethal alleles. Apparently there are a large number of independently segregating lethal alleles in the pine population. Thus, the likelihood that two individuals will have matching heterozygous lethal alleles is very low. However, with any degree of inbreeding, and in particular with self-pollination, there is a relatively high probability that a homozygous combination of lethal alleles will occur at one or more loci. Individual embryos that have homozygous lethals abort soon after fertilization. If only one fertilization occurs in the ovule, embryonic abortion results in an empty seed at cone maturity. However, because pines are polyembryonic, empty seeds occur only in ovules where all the embryos in a given ovule have aborted. Therefore embryonic lethal alleles are not a major

cause of empty seeds when controlled pollinations are completed among unrelated parents. When controlled pollinations are made among related parents, some reduction in filled seed yields should be expected.

If you have good insect protection, no fungal problems, and no inbreeding and you still get a large number of empty seeds, the probable cause is low pollen vigor. We first became aware of this problem in loblolly pine when we tested storage methods for pollen (Matthews and Bramlett 1983). We noted that pollen stored in a desiccator produced similar numbers of fully developed seeds as pollen stored under vacuum or as fresh pollen. The number of filled seeds, however, was greatly reduced after pollinations with the desiccator-stored pollen. In vitro germination tests of the pollen prior to controlled pollinations indicated that the viability of the desiccator-stored pollen was as good as the vacuum-stored or fresh pollen. Pollination with the viable pollen stimulated the ovules to develop archegonia and fully developed seedcoats, but the pollen apparently was not vigorous enough to complete fertilization. Without fertilization, the development of the gametophyte stopped and the result was an empty seed.

We confirmed these results in a similar test using different pollen lots (Bramlett and Matthews 1991). Thus, low pollen vigor shows up as an increase in the number of empty seed and apparently is associated with deterioration during storage. Tree breeders who observe large numbers of empty seeds should check their pollen storage procedures for possible reductions in pollen vigor. Jett et al. (1993) have described the currently recommended procedures for pollen extraction, testing and storage.

Conelet survival

Only two causes of conelet losses may be attributed directly to the pollination procedure (Table 2). First, if conelets do not receive adequate pollen, they may abort. Just how many pollen grains are required for the female parent to retain conelets is not known. Some clones will hold unpollinated cones to maturity without producing any viable seeds. In other clones, poor pollination causes some abortion of conelets after pollination.

The second pollination-related loss of conelets is from damage caused by the bag. The developing female flowers are delicate structures and any buffeting from wind or rubbing against the bag (particularly sausage casing) can cause light to severe damage. When the damage is severe, the conelet usually dies.

All other causes of conelet mortality are unrelated to the pollination process. These causes include damage from insects, fungi, freezing temperatures, and high winds. Tree breeders should take adequate measures to minimize these losses but total elimination is not possible. A reasonable goal is to keep conelet losses to less than 10 percent.

Small mature cones

When pine flowers are not adequately pollinated, the mature cone is noticeably smaller than open-pollinated cones on the same tree (table 2). Size is reduced because the cone scales associated with unpollinated ovules are

stunted. If small cones are observed after controlled pollinations, pollen quantity, distribution, or viability should be checked for improvements.

Table 2.--Diagnostic observations, probable causes and procedures to check to reduce losses of cones from controlled pollinations.

<u>Diagnostic observation</u>	<u>:</u>	<u>Probable causes</u>	<u>:</u>	<u>Procedures to reduce losses</u>
A. Conelet survival (< 90%)		1. Insect damage	.	Check pest management program
		2. Bag damage	.	Check bagging procedure
		3. Inadequate pollination	.	Check timing of pollen application
			.	Check application amount pollen applied
			.	Check pollinator for distribution in bag
		4. Low pollen viability	.	Complete <u>in vitro</u> germination test
			.	Check collection, and storage procedure
		5. Freezing temperatures	.	Avoid frost pockets
		6. Fungi	.	Avoid branches with pitch canker
		7. Wind	.	No known prevention
B. Small mature cones (25% smaller than open-pollinated cones.		1. Inadequate pollination	.	Check timing of pollen application
			.	Check application amount pollen applied
			.	Check pollinator for distribution in bag
		2. Low pollen viability	.	Complete in vitro germination test
			.	Check collection, and storage procedure
C. Cone Survival (< 75% of flower crop)		1. Insects	.	Check pest management program
		2. Fungi	.	Reduce pitch canker in orchard

Poor cone survival (year 2)

Poor cone survival during the second year is not related to the pollination procedure (table 2). Insect damage is the most likely cause of second-year cone mortality, but fungi are also known to cause cone mortality. Squirrels can be a problem in some orchards. If these problems are reducing cone and seed yields from controlled pollinations, the pest management program should be evaluated to reduce cones losses.

METHODS TO ASSESS POLLINATION PROBLEMS

Many causes of reduced seed and cone yields in seed orchards have been identified. It is important to know which ones are caused by poor or incomplete controlled pollination. Perhaps the simplest way is to collect at least five open-pollinated cones from the same tree for comparison. The control-pollinated cones should be extracted first. If the seeds are extracted in a bulk lot, the total number of seed should be divided by the total number of cones. When insect-damaged cones are in the cone collection, estimate the amount of seed each damaged cone contributed (for example 25%-75%).

Next, separate filled from empty seeds, and count the number of filled seed and empty seed per cone. Air blowers or flotation systems are satisfactory for separating the seeds. Estimate the number of aborted ovules by subtracting the total number of developed seeds per cone from the seed potential. If you do not have seed potential data for each clone, use 155 for loblolly, 170 for slash and 90 for shortleaf and Virginia pine (Bramlett et al. 1977).

If seed yields are 80-100^{1/} filled seeds per cone and the cone survival is also high (> 75%), the controlled pollination should provide adequate seed for progeny testing. If seed yields do not meet expectations, compare control-pollinated and open-pollinated and seed yields per cone. Of all the diagnostic observations, the number of first-year aborted ovules and the percent of empty seeds per cone are the most revealing. You do not want to try to correct a pollination problem if in fact you have insect or other causes of seed losses. If the open-pollinated cones have fewer first-year aborted ovules or a lower percentage of empty seeds, the difference between the control- and open-pollinated cones is an estimate of the "pollination effect."

The next step is to examine the suspected causes in tables 1 and 2 for the diagnostic observation. It is quite likely that several causes are contributing to the loss. Then, you must decide whether the loss is large enough to justify corrective action. In general, losses associated with poor pollination can be corrected.

CONCLUSION

There is no apparent biological reason why tree breeders cannot produce an average of 80-100 filled seeds per cone and 75% cone survival from controlled pollinations in loblolly or slash pine. To achieve these goals, care must be taken in the collection, extraction, processing, and storage of pine pollen. High-quality pollen must be correctly applied to bagged female flowers. And the flowers, conelets, and cones must be protected to maturity. If these goals can be consistently achieved, tree breeding can rapidly move forward with progeny testing and continued breeding for future generations of genetically improved southern pines.

^{1/}For loblolly and slash pine; use 40-50 filled seeds for shortleaf and Virginia pine.

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A PROPOSAL FOR A SOUTHERN FOREST GENE CONSERVATION PLAN

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The conservation of our nation's forest genetic resources has lagged behind that of agricultural species. The practice of scientific forestry has maintained the genetic diversity of the economically valuable forest tree species. Because of this the forestry community has become complacent about ensuring the long term conservation of our forest genetic resources. If forest genetic resource managers do not take an active role in developing tree gene conservation strategies, someone with opposing views will step in and do it for us.

The Southern region is blessed with an abundance of forest genetic resources and the trained personnel to direct their future use. The members of the Southern Tree Improvement Conference are sufficiently organized to develop a Southern Forest Gene conservation Plan for southern tree species. This proposal involves developing management strategies for long term in situ and ex situ gene conservation with an accompanied database management system. This regional gene conservation plan could integrate with other gene conservation networks to effectively conserve and maintain the total tree gene pool.

Keywords: gene conservation, forest genetic resources, in situ, ex situ

INTRODUCTION

The forested areas of the North American continent comprise about 734 million hectares or 40% of its land area. (World Resources Institute, 1988). The practice of forestry has maintained the gene pool of our economically valuable forest tree species, therefore many native tree species are not close to extinction. Because of this the forestry community has become complacent about ensuring the long term conservation of our forest genetic resources. Tree seed collections have primarily been for short-or-medium term storage for use in afforestation or reforestation activities. Few programs have long term objectives.

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Genetic Variation

Forest genetic resources refer not to the seed itself but to the genetic information found in the chromosomes and associated subcellular structures(Kloppenber, Jr. , 1988). To ensure the conservation of these forest genetic resources, knowledge of the diversity and distribution of genes in a species' population is crucial to genetic management(National Research Council, 1991). Gene flow determines the geographic scale over which species' populations may be differentiated from each other. Mating systems and the geographical range of species have significant effects on the level of genetic variation among species' population. Differences between pollen and seed migration often coincide with selective differences which affects genetic diversity, genetic drift and the probability distributions of unique alleles(Namkoong and Gregorius, 1985).

These nonrandom arrangements of genotypes impose a structure on a population. Understanding genetic structure in a tree species will make it possible to sample the genetic diversity of populations correctly.

Man's intervention can also increase genetic diversity through forest management, breeding, and biotechnology. Reforestation and afforestation can extend the range of species into areas where different selection pressures favor unique alleles. Breeding can create greater diversity among populations within species(National Research Council, 1991). Biotechnology does not replace traditional plant breeding, rather it extends the gene pools of species by overcoming incompatibility barriers of reproduction(Kloppenburg, Jr., 1988).

Managing Forest Genetic Resources

Managing forest genetic resources involves developing overall strategies, applying methodologies, developing new technologies, and coordinating organizational efforts(National Research Council, 1991). The forestry community is fortunate in that we can tie into the networks already established by agricultural research. The advantage of using the networks in place is that existing facilities and staff can be used rather than erecting new structures and adding more bureaucracy.

The Southern forest genetics managers already have a network in place and it is called the Southern Forest Tree Improvement Conference(SFTIC). SFTIC is classified as an information exchange network which is used as a forum to exchange information among the collaborative forest genetics research networks and state and federal forestry agencies(Plucknett et. al. 1990). As a network, SFTIC has already developed the rules of governance, leadership, and management(Plucknett et. al., 1990). SFTIC is ideally suited to interact with other agricultural networks in developing and implementing a Southern Forest Gene Conservation Plan.

SOUTHERN FOREST GENE CONSERVATION PLAN

The main purpose of this proposal for a Southern Forest Gene Conservation Plan is to preserve the excellent work on the genetic management of Southern pines by the collaborative forest genetics research networks, and the state and federal forestry agencies. This regional gene conservation plan is being proposed because the southern forest genetics managers are well organized and the opportunity is strong for establishing leadership in developing regional and national systems of forest tree gene conservation. The proposal is not meant to create more work for everyone, but to organize and preserve the legacy of the southern forest genetics managers. The major objective of forest tree improvement has been to provide genetically improved material for reforestation. In the short term, this objective has been met. I am only suggesting that a sample of the southern pines' genetic base be conserved in the event that the southern forest genetics managers need to use it in the future.

In situ conservation

There are 2 ways to conserve genetic material: in situ and ex situ(National Research Council, 1991). In situ conservation and ex situ conservation are complementary strategies. In situ conservation preserves the population and the evolutionary processes that enable the population to adapt(National Research Council, 1990). Good examples of in situ conservation are national parks, state parks, nature conservancy landholdings, wildlife refuges, wilderness areas, and multiple-use management areas. The problem with in situ conservation of forest genetic resources is that genetic variation and determining an effective population size for a viable gene pool must be considered rather than the size of the area.(Roche, 1975). Designing tree reserves and managing natural or artificial stands requires an understanding of the reproductive systems of the trees being conserved(National Research Council, 1991). In my opinion, in situ conservation is beyond the scope of SFTIC, because the organization's membership is more geared to scientific advancements than legislative and managerial processes.

Ex situ conservation

In contrast, ex situ conservation preserves the genetic material as samples of the population that are readily available for use. Examples of ex situ conservation are seed, pollen, tissue culture, DNA fragments, clone banks, seed orchards, seed production areas, provenance studies, and progeny tests. Ex situ conservation is within the scope of SFTIC, because SFTIC's members do have direct control over the ex situ elements of conservation. Therefore, the proposal for a Southern Forest Gene Conservation Plan is exclusively concentrated on ex situ conservation(Figure 1). The ex situ genetic material is already available and could easily be organized for conservation.

Collections

Ex situ conservation in agricultural crops is usually organized into 3 kinds of collections according to their function: active collections, breeders' collections, and base collections(National Research Council, 1991). Active collections include the genetic material in seed orchards, clone banks, progeny tests, and provenance tests. These are the primary sites for distribution, evaluation, and general management. Active collections are usually maintained until they outlive their usefulness.

Breeders' collections consist of inbred lines, superior varieties, elite lines, and hybrids mostly found in agricultural crops(National Research Council, 1991). It denotes the germplasm used most frequently by plant breeders. In forestry, breeders' collections would consist of the breeding population of the species. The breeders' collections are a subset of the active collections(National Research Council, 1991).

Base collections are backup reserves of active collections held under condition of long term storage(National Research Council, 1991). Long term storage is defined as spanning a period of time longer than one rotation (Anonymous, 1984). Base collections insure against total loss in the event the active collections are destroyed(Kloppenburger, Jr., 1988). Base collections can encompass the range of genetic diversity within a species or just be a subset of its range.(National Research Council, 1991).

Since long term preservation is the objective of base collections, they are usually kept of species with true orthodox seeds(Roche, 1975). Pine seeds are classified as orthodox(Schopmeyer, 1974). True orthodox seeds can be stored for relatively long periods at sub-freezing temperatures if their moisture contents are reduced to below 10%(Bonner, 1990). This storage environment is the preferred standard for long term storage for genetic resources conservation, because it is technically achievable at a reasonable cost while insuring the reduction in viability of orthodox seeds occurs very slowly(IBMGR, 1985).

Long Term Storage

There are a few studies on the effects of long term storage of forest tree species. *Pinus resinosa* seed maintained high germination stored for 42 years and *Pinus elliottii* seed had 66% germination after 50 years in cold storage(Bonner, 1990). Samples of true orthodox tree seeds, including some firs and pines, have been stored up to 6 months in liquid nitrogen(LN₂) without adverse effects(Bonner, 1990). There is no evidence to suggest that long term storage of true orthodox tree seeds will not be just as successful as it has been with agricultural seeds.

Genetic changes during long term seed storage are possible, because the loss of vigor could lead to genetic drift(Bonner, 1990). It is important at the onset of storage to provide enough seed for each accession to reduce the

effects of genetic drift. Seed can be replenished in a base collection when the viability begins to decrease. Genetic change may occur during replenishment through inadvertent selection and seed aging(National Research Council, 1991). Gene frequency comparisons before and after seed replenishment of the base collection could be measured using electrophoresis or restriction fragment length polymorphisms(RFLP).

Methods of storage that reduce the frequency of seed replenishment are very important. For long term seed storage, the seed storage facility needs good environmental controls to keep the temperature and moisture constant(Bonner, 1990). Most organizations do not have up-to-date seed storage facilities or the storage capacity to store seed at low temperatures for long periods of time. One such facility does exist within the Department of Agriculture. It is called the National Seed Storage Laboratory(NSSL) and it is located in Ft. Collins, Colorado. A new 66,000 square foot addition was recently built to address the future demand of seed conservation. A portion of the facility has been designated for forestry tree seed(personal communications with Henry L. Shands, Associate Deputy Administrator, Genetic Resources of USDA).

National Seed Storage Laboratory

I would like to propose as part of the Southern Forest Gene Conservation Plan that seed samples be stored at the National Seed Storage Laboratory(NSSL)as base collections. These base collections would be placed in heat sealed packets and serve as a backup to active collections stored or planted at another location. The Center for Plant Conservation (CPC), an association of U.S. botanical gardens, maintains rare and endangered U.S. plant species(National Research Council, 1991). The National Plant Germplasm System, a part of the USDA, provides backup storage of seeds for CPC's collections at its western agricultural research station and at the National Seed Storage Laboratory(National Research Council, 1991). Just like the CPC, SFTIC members could set up a memorandum of understanding with the USDA to house southern forest tree seed at the National Seed Storage Laboratory. There is no monetary charge for storing seed at NSSL(personal communications with Henry L. Shands, Associate Deputy Administrator, Genetic Resources of USDA).

The NSSL does not replenish, evaluate, enhance, or distribute germplasm as part of its mandate(National Research Council, 1991). Eventhough, the NSSL may have the capacity to store seed, the facility does not have the personnel or expertise to prepare the tree seed for storage, to test tree seeds, and maintain a record keeping system. The National Tree Seed Laboratory(NTSL) could act as a clearinghouse by providing their expertise and services in preparing the seed for storage. The Southern forest genetics managers could send the seed for the base collections to the seedlab. The NTSL personnel would check the moisture content before packaging the seed in the heat sealed packets, and log the seed samples into a database. Periodic germination tests on the base collections could also be performed by seedlab personnel. The documentation of the active and breeders' collections could be combined with the base collections' database or kept in separate databases.

It would be the responsibility of the SFTIC membership to establish the protocols on the amount of seed in each sample needed to maintain genetic integrity, establish viability standards, parameters of storage, timing of germination retesting, and database maintenance. An ad hoc committee within SFTIC could be formed to iron out the details of a Southern Forest Gene Conservation Plan. Once the conservation of the southern pines was underway, more southern tree species could be included in the conservation plan if desired by the group. Individual organizations could formulate their own germplasm conservation plan if there were no consensus within the SFTIC membership. Each organization would have to negotiate with the National Seed Storage Laboratory for seed storage.

Why bother?

The purpose of this proposal is to preserve the legacy of the southern forest genetics managers for future use. Seeds are just seeds when produced by trees, but they become germplasm when gathered to conserve genetic diversity, develop a breeding program, or preserve specific genetically controlled traits(National Research Council, 1991). The phenomenal agricultural productivity of the U.S. has come from using germplasm to improve crops genetically(National Research Council, 1991). The National Plant Germplasm System, developed by the USDA, insures that agricultural scientists have genetic material to work with in the future(National Research Council, 1991).

Similarly, the practice of forestry has conserved the major forest tree species by regenerating tree species after timber harvesting. This in situ conservation has insured a ready supply of timber for products that have fueled U.S. economic expansion and stability.

Society will continue to need the services and products from the forest. As other countries deforest their landholdings, there will be more pressure on the U.S. forests to meet not just the nation's demand for forest products, but a global demand for wood products. With the environmental controversy concerning western forests, more attention will be focused on the southern region to provide more of the wood supply. Sustaining forest productivity will require continued use and access to a broad diversity of germplasm. Managing forest genetics resources will become a strategic necessity for the U.S.(National Research Council, 1991).

An ex situ germplasm management plan may provide a buffer against the loss of genetic management through personnel and organizational changes. New personnel may lose interest in past efforts and abandon projects in pursuit of their own ideas. Some of the collaborative research networks may be disbanded due to fiscal problems or loss of interest. Forestry schools could be closed in the future due to lack of funding and decreasing enrollments.. Genetic material could be lost, misplaced, or abandoned.

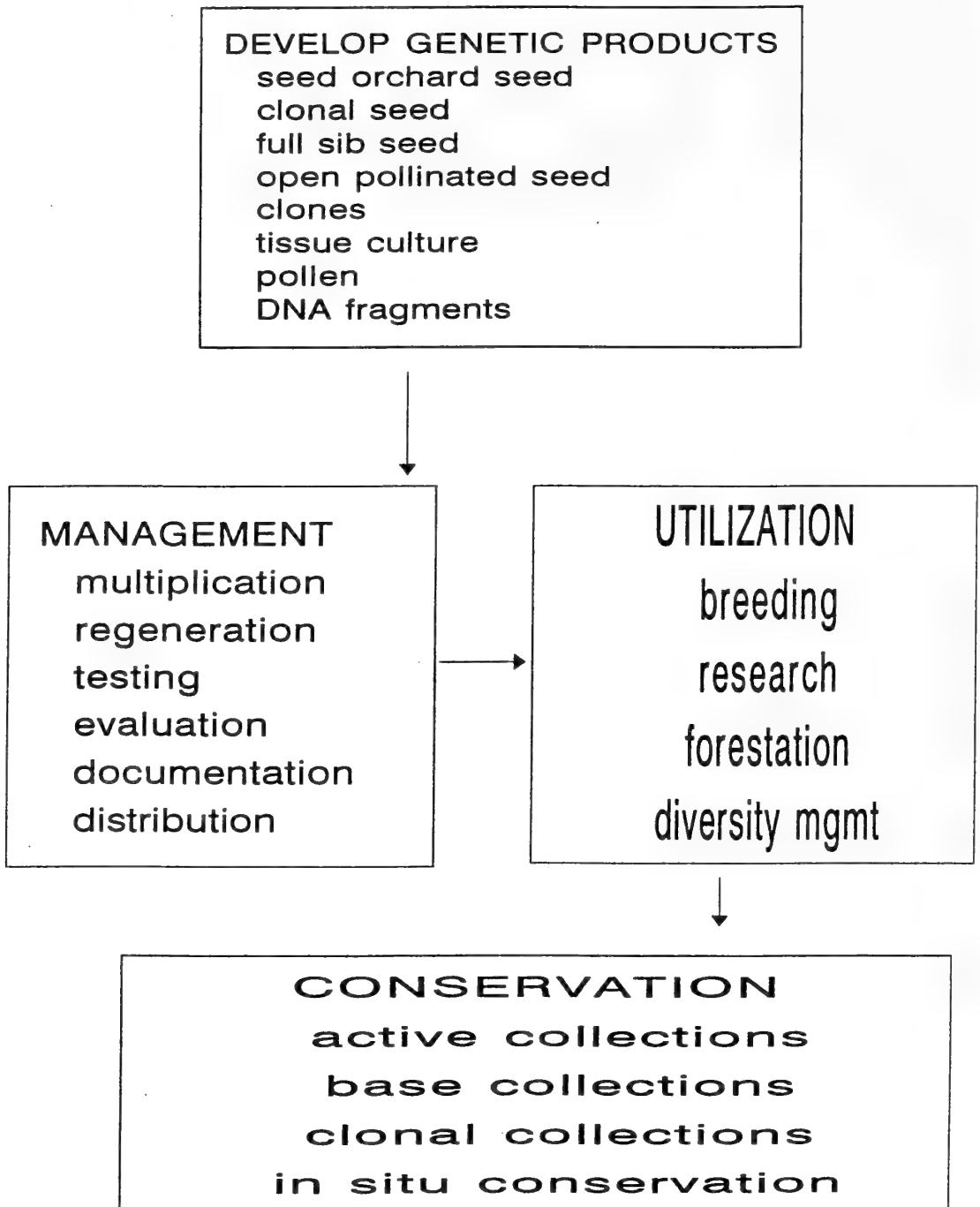
The genetic material could also be destroyed without the proper storage or correct seed conditioning. Proper environmental controls and monitoring equipment of a storage facility are needed to store seed without loss of viability. Storing southern pine species' base collections at a facility, such as the National Seed Storage Laboratory, would make it easier to track the seed's viability over a long time period, so that the base collections could be replenished when their viability fell below set parameters.

Conclusion

If forest genetic resource managers do not develop a germplasm system to conserve the forest tree resources, who will? They are the most knowledgeable concerning the conservation of the region's forest genetic resources, and have the most access to the collection within an ex situ germplasm management plan.

It is my hope that such a plan will be developed and implemented within the southern region. The southern region could lead the way for the rest of the nation in gene conservation. The southern forest gene conservation plan could be expanded or duplicated to include the other regions of the U.S.

EX SITU GERMPLASM MANAGEMENT PROGRAM



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RESEARCH AND MANAGEMENT IN A YOUNG NORTHERN RED OAK SEEDLING SEED ORCHARD

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Abstract.--A northern red oak progeny test was thinned at age 15 to produce a seedling seed orchard. Studies were initiated to determine relationships between acorn production and seed source. Acorn production was observed in 1984-1986 and 1989-1992. Family differences were observed in reproductive maturity. Large differences in size of acorns from the same tree were observed. Many acorns appeared to be extensively damaged by insects. A progressive loss of flowers, acornets and acorns was observed on different genotypes from 1989-1991 as the acorns matured. Oak anthracnose disease has been intermittently damaging in the orchard when environmental conditions are favorable for infection. Two different fungi, alone and in combination, have been consistently isolated and confirmed pathogenic. Insect populations were sampled in 1992. Population levels of different species fluctuated throughout the growing season.

Keywords: northern red oak, *Quercus rubra*, seed orchard, acorn, pest management, first-order lateral roots

INTRODUCTION

Many studies have been conducted on cultural practices and pest management in seed orchards of coniferous species (cf. Bramlett 1991). In contrast, management guidelines for orchards containing North American oak (*Quercus*) species are virtually unknown. Past difficulties in vegetative propagation of oak species, either through grafting, rooted cuttings or tissue

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culture, have limited efforts in constructing seed orchards. Additionally, in the few grafted orchards that exist, the growth and acorn production of grafted clones is extremely variable (Schlarbaum *et al.* 1993). Seedling seed orchards are an alternative for production of genetically improved seed, although genetic gains can be lower in comparison to clonal orchards. In 1987, a progeny test of northern red oak (*Quercus rubra* L.) on the Watauga Ranger District, Cherokee National Forest, was converted to a seedling seed orchard (La Farge and Lewis 1987). This seed orchard was constructed to provide acorns for reforestation efforts in the U. S. Forest Service's Southern Region. Since construction, the orchard has been the subject of various studies on acorn production, pest management and harvesting procedures. In this paper, a brief description of completed and ongoing research will be presented. More detailed accounts of the investigations will appear elsewhere in individual publications.

ORCHARD DESCRIPTION

The orchard is located in the southern Appalachian foothills, near Elizabethton, Tennessee (Lat. 36°07"N, Long. 82°00"W, Elev. 610 m) and is 5.9 hectares in size. The site was an old agricultural field that was originally covered by weeds and fescue and was prepared for planting by plowing and disking. The progeny test was established in 1973 by the Tennessee Valley Authority (TVA). It consisted of 220 open-pollinated families from acorns collected primarily throughout the Tennessee River Valley in 1971. Some mother trees were TVA phenotypic selections, although the majority of families came from trees that had acorns and were considered as generally good phenotypes. Initial survival of the seedlings was approximately 60 percent. In 1982, the USDA Forest Service, Southern Region attained control of the plantation as the TVA tree improvement program had been discontinued. By age 13, grass and weed competition had lowered survival to 44.4 percent. Additionally, the plantation was periodically browsed by deer.

The planting was evaluated in 1985 for six traits: height, diameter, straightness, forking, insect defoliation and apical dominance. The study was partially thinned/rogued in 1987. Only a relatively low number of families (12) were designated for roguing, because of the young age. Subsequent maintenance has consisted of mowing and spraying around the base of the trees with an herbicide to control grass competition. A more detailed description of the orchard site and the criteria and analyses used for construction of the seed orchard has been published by La Farge and Lewis (1987).

ACORN PRODUCTION AND QUALITY

Acorn production was assessed in 1984-1986, prior to thinning, and in 1989-1992. No collections were made in 1987 and 1988 due to the activities associated with the conversion. Production was relatively limited in the 1984-1986 period (Table 1). In contrast,

a large number of families bore acorns in 1989.

Table 1. Acorn production in the northern red oak seed orchard, Watagua Ranger District, Cherokee National Forest.

<u>Year</u>	<u>Number of Families</u>	<u>Total Number of Trees</u>
1984	5	unknown
1985	22	26
1986	15	unknown
1989	87	132
1990	11	12
1991	74	98
1992	86	139

Acorn production in 1990 was relatively low, but rose dramatically in 1991. Data in Table 1 suggest that production could be episodic, but environmental factors or insect damage could also be possible reasons for fluctuation. Family differences were present among seed sources for acorn production. Acorn production within a family, however, was not consistent. There was great variation among individual trees in the number of acorns produced.

In 1992, acorn collections from individual trees were made on a daily basis in order to study relationships between quality and date of drop and to determine the length of time required to complete acorn drop. Nets were placed under 66 trees that produced moderate to large crops of acorns. From September 26 until October 9, acorns were collected each day and kept in separate bags. After October 9, it became apparent that predation by squirrels was causing significant losses, so bulk collections were made. The acorns were knocked off the branches using a wooden pole.

Acorn quality was assessed by floating each daily collection in water initially after collection and later, prior to sowing. Floating acorns were discarded from the initial immersion, but reserved from the second immersion for insect studies (see below). The proportion of floating acorns was higher in the earlier collection dates. Sinking acorns ("sinkers") are generally presumed to be good acorns, i.e., capable of producing a seedling. Destructive and X-ray inspections of sinkers, however, revealed that a high proportion, e.g., up to 90 percent, had significant insect damage.

Viable acorns of ten families were planted at two locations to study first-order lateral root (FOLR) distributions and heritability. Resulting seedlings will be individually graded for FOLR and other growth characteristics (Ruehle and Kormanik 1986). Seedlings will be planted in experimental designs at different locations for performance evaluation over time.

Acorn size variability within an individual tree collection was present. Surprisingly, as much as fourfold size differences

were found. The smaller acorns had the same general shape and cap characteristics as the larger acorns and appeared to be normal. This variation was not observed in all seedlots. For some families, the acorns were sorted and the smaller acorns were kept and grown separately.

DISEASE

The orchard was surveyed for stem canker diseases in 1988, but it was not until 1990 that any potentially serious diseases were recognized. In July, 1990, a foliage disorder was observed that was initially thought to have been caused by the herbicide glyphosate used for weed control around each tree. However, the herbicide hypothesis was discounted after the orchard was thoroughly inspected. Damaged foliage of one tree was often intermixed with healthy foliage of another, with both presumably exposed to the same herbicide concentration and environment. Symptoms were concentrated in the lower half of the live crown and were characteristic of an anthracnose-type foliage disease. These symptoms included leaf cupping and curling, marginal necrosis, an irregular leaf blotch, and a discrete pinhole leaf spot. Shoot dieback and epicormic sprouting were occasionally found on some affected branches. Leaf curling and cupping indicated the initial damage was occurring early in spring during leaf expansion. Despite these indications, oak anthracnose was discounted due to the low susceptibility of red oaks relative to white oak, and the absence of damage on white oaks in the immediate vicinity of the orchard. Laboratory culturing of samples collected in August, 1990 was unsuccessful in isolating any known leaf pathogen, apparently due to late sampling.

The disease recurred in 1991, but culturing of late June samples was also unsuccessful. Symptoms were detected in early May, 1992 and this time two fungi were consistently isolated. One bears a close resemblance to dogwood anthracnose fungus in culture and has been tentatively identified as Discula quercina. The other fungi has been tentatively identified as Colletotrichum acutatum. The pathogenicity of both fungi has been confirmed in laboratory inoculations of northern red oak seedlings.

Disease incidence and severity was greater in 1990 and 1991 than 1992. Heavy infection of some of the smaller trees in consecutive years probably was a significant factor in their mortality. Repeated defoliation of the lower limbs of larger trees may have resulted in some limb mortality, but the overall effect of the disease on these trees was small.

INSECT STUDIES

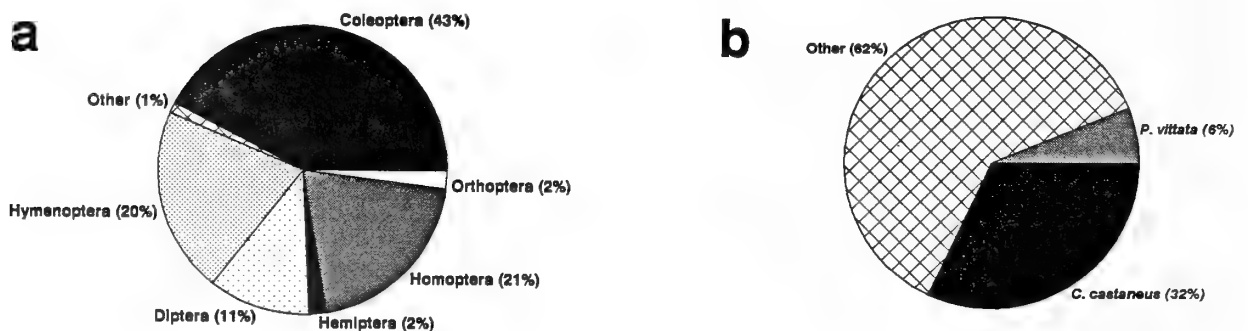
The importance of pest management in conifer seed orchards has been well documented (DeBarr and Barber 1975, Bramlett 1991). Studies have shown that oaks harbor a number of insects that have the potential to damage developing acorns either in the adult or

larval stage (cf. Solomon et al. 1987). Correspondingly, studies were initiated to determine if flower/acorn loss was occurring prior to acorn maturity, define the diversity of insect species in the orchard, understand relationships of insects to acorn crop size and quality, and evaluate the effectiveness of spraying for protection.

In 1989, 20 trees from 18 different families were selected for studies on flower, acornnet (1st year acorn), and acorn loss prior to maturity. Branches were tagged in each tree and individual flowers/acornnets/acorns were counted in early spring, mid-summer and fall. Two complete developmental cycles (flower to mature acorn) were observed, 1989-1990 and 1990-1991. Approximately 4 and 20 percent of the flowers developed into acorns in each respective cycle. These percentages are similar to what can be expected in a coniferous seed orchard without pest control.

A study on insect diversity and population levels was initiated in 1992. Five families were selected for the study based upon previous acorn production information. Four families had some trees that produced acorns in previous years; one family had no history of production. The trees were sprayed with Ansana™, and the resulting dead insects were collected on nets. Spraying began in late March immediately prior to budbreak and continued through early October at two-week intervals. A total of 13,052 insects was collected in 1992. Gross sorting revealed that the majority of insects (99 percent) could be classified into six orders (Figure 1a). Two insect species dominated the collection, in terms of

Figure 1. Insect diversity in 1992 orchard samples. A total of 13,052 insects was collected. (a) Percentages of insects in different taxonomic orders. (b) Percentages of Asiatic oak weevil (Cyrtepistomus castaneus) and an oak treehopper (Platycotis vittata) in total collection.



numbers. The Asiatic oak weevil, Cyrtepistomus castaneus, and an oak treehopper, Platycotis vittata, comprised 32 and 6 percent,

respectively, of the total number of insects (Figure 1b). Each insect has the potential to cause significant damage to acorn crops. Asiatic oak weevils may damage acornets during the first year of growth. Platycotis has sucking mouthparts and could damage flowers and acornets.

Insect damage was evident in mature acorns collected in 1992. Samples of acorns that floated were placed in screened pots containing soil. Weevil larvae emerged from the acorns and migrated to the bottom of the container where they will pupate and emerge in late 1993. Species identification will be made after emergence.

FUTURE STUDIES

The orchard will be surveyed in the early spring, 1993, for acorns. Results will be compared to actual acorn counts to evaluate the reliability of visual survey for predicting production. The orchard will also be surveyed for male catkin production. Sister plantations in west Tennessee and Kentucky will be observed for acorn production to calculate a heritability estimate.

Daily collections of acorns will be made in 1993 by individual trees and kept separately. Daily acorn counts will be used to construct an acorn maturation distribution for each family. Acorn drop will be correlated with weather data to determine any critical environmental factors that influence maturation/drop. Acorn quality will be assessed by floating, destructive sampling and X-ray analyses. After processing, the acorns of selected trees/families will be planted separately according to day of collection. Resulting seedlings will be evaluated for FOLR numbers to determine if there is a relationships between the number of seedlings with high FOLR numbers and seed collection (maturation) date. All data associated with the collection will be used to develop guidelines for harvesting high quality acorns.

The insect diversity study will continue through 1993. Species emergence and population levels for 1992 and 1993 collections will be compared to environmental factors, e.g., growing degree days, to develop predictive emergence models. Additional studies will be made on specific insects suspected of causing flower/acorn damage. Emergence traps for thrips (Thripidae), Asiatic oak weevil and acorn weevils will be placed under trees of five families. Sleeve cages will be placed over feeding populations of Asiatic oak weevils and Platycotis to detect damage or mortality to acornets and acorns. A number of trees (30) have been selected for spraying to protect the current acorn crop and the 1994 crop. The quality of acorns from these trees will be assessed after fall collection.

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MERITS OF USING MECHANICAL TREATMENTS TO STIMULATE CONE PRODUCTION OF SLASH AND LONGLEAF PINES

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Abstract. Four mechanical treatments (untreated, partial girdling in the spring, partial girdling in summer, and banding in spring) stimulated cone production of pole-sized slash and longleaf pines. A 2- to 3-fold increase in slash pine seed production was limited to the first crop originating after the treatments were applied. However, the treatments killed half the longleaf pines, preventing any overall increase in seed productivity. Although mechanical stimulation of cone production has been reported for decades, the method cannot be generally recommended for use in slash and longleaf pine seed orchards where the value of individual trees is great and where susceptibility to injury may vary by species and clonal family.

Keywords: Pinus elliottii Engelm., P. palustris Mill., seed orchard, girdling, seed quality.

INTRODUCTION

In the last several decades numerous studies have evaluated ways to increase the seed production of various species of southern pines. Loblolly pine (Pinus taeda L.) has been the focus of much of this research because it is the most widely grown species in southern pine seed orchards. Crown release, fertilization, irrigation, and, to a lesser extent, injury and chemicals have been used to stimulate early and heavy flowering. Wheeler and Bramlett (1991) recently reported flower stimulation of loblolly pines from girdling and gibberellin (GA_{4/7}) treatments. Most of the response resulted from the girdling treatments.

The use of injury to stimulate flowering of fruit trees was documented as early as the 18th century. Injuring forest trees to stimulate seed production was attempted in the early 1920's. Since these early tests, injury has been researched extensively in many coniferous species, but it has been used infrequently in seed orchards for fear of harming valuable trees and inconsistency of response. There also has been concern that injury will weaken trees, resulting in greater susceptibility to insects or mortality.

The purpose of the study reported here was to determine the response of slash pine (P. elliottii Engelm.) and longleaf pine (P. palustris Mill.) to girdling and strangulation. The treatments were evaluated by measuring cone production, seed yield and quality, and tree growth and mortality.

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METHODS

Four treatments were tested: (1) partial girdling in spring and (2) in summer, (3) banding with wire in spring, and (4) an untreated control, applied to 23-year-old slash and longleaf pines located on the Palustris Experimental Forest near Alexandria, Louisiana. Study trees ranged in diameter from 11.0 to 12.9 inches and were selected for uniformity in crown size, vigor, and past cone production. Treatments were replicated 10 times in a randomized block design. Trees were grouped into blocks of four on the basis of promixity and treatments were assigned at random within each block. Analyses of variance and orthogonal comparisons were used to test treatment differences at the statistical significance level of 0.05.

Girdling was done using a girdling machine that cut through the bark and cambium. The girdles consisted of two semi-circular cuts approximately 1-inch wide. They were 4 inches apart vertically and overlapped 1 inch on each end. Girdling was done in early April and late June. Following treatment, an insecticide was applied to the wounded areas to reduce bark beetle attack.

Wire bands for the strangulation treatment were applied in early April and consisted of three strands of #9 wire. Loose bark was removed so that the wire made uniform contact around the trees.

At the time of establishment, d.b.h., total height, and length of live crown were measured. Also, previous cone production was estimated. Slash pines were remeasured after 2 1/2 years. Each year after treatment, cone production was estimated in July or August using one-position, binocular counts (Hoekstra 1960).

In the second-year after treatment, 10 cones were collected from each slash pine tree to evaluate seed yield per cone and seed viability. Due to the mortality of the longleaf trees, cones were not collected for this species. All empty seeds were removed by flotation (Barnett 1971) and 50 sound seeds per tree were tested following standard laboratory procedures (AOSA 1980).

RESULTS

Cone Production

Cone production was not affected by injury until the second year after treatment (+2). In that year, slash pine cone production was increased 62 percent over the control by banding, 133 percent by spring girdling, and 200 percent by summer girdling (Table 1). Individual degree of freedom comparisons showed that injured trees yielded significantly more cones than control trees, but that there were no significant differences in yields between banding and girdling or between spring and summer girdling. There were an estimated 21 cones per tree for the control and an average of 49 cones per injured tree. In the following year (+3), counts for slash pine ranged from 50 cones per control tree to 99 cones per tree for the summer

girdling treatment, but differences were not statistically significant (Table 1). It does appear that banding may have a greater lag in response than girdling.

Table 1. Average cone production by treatment, year^x, and species, based on one-position binocular counts.

Species and treatment	Cones per tree per year				
	-1	0	+1	+2	+3
<u>Slash pine</u>					
Banding	26	21	50	34b ^y	82
Spring girdling	30	16	43	49b	70
Summer girdling	19	10	36	63b	99
Untreated control	30	19	43	21a	50
Average	26	16	43	42	75
<u>Longleaf pine</u>					
Banding	50	10	55	5	22(8) ^z
Spring girdling	53	11	31(7)	8(5)	3(1)
Summer girdling	62	18	52	18(5)	10(2)
Untreated control	41	10	39	9	22
Average	49	12	44	10	14

^xThe year of treatment application is indicated by 0, years before or after this year are shown by (-) or (+).

^yValues followed by different letters are significantly different at the 0.05 level.

^zFigures in parentheses are the number of living trees used for production estimates. Dead trees were assigned a value of zero in computing means for treatments.

Half of the girdled longleaf pines died before the cone counts in year +2. Therefore, analysis of the +2 and +3 year data compared only banding and the control. There were no significant differences between these treatments in either year.

Average values for living longleaf pine trees indicate that both spring and summer girdling may have increased cone production, especially in year +2. On this basis, spring girdling increased production 78 percent in year +2 and 36 percent in year +3. Summer girdling boosted production 300 percent and 127 percent, respectively, in the 2 years. These increases roughly parallel results with slash pine which suffered no mortality during the study, but mortality precluded a statistical analysis. However, treatments actually decreased net production if mortality is considered (Table 1). Average cone counts for living longleaf trees were as follows:

Treatment	Year	
	+2	+3
	Number	
Banding	5	28
Spring girdling	16	30
Summer girdling	36	50
Untreated control	9	22

Seed Yield and Germination

The influence of increased slash pine cone production on numbers of sound seeds per cone and seed viability was determined for cones collected in year +2, the only year there were significant increases in cone production. The seed yields were low, averaging only 13 per cone (Table 2). There was a trend toward total higher seed yield from injured trees, but the differences were not significant. Nevertheless, the increased cone yield increased seed production on injured trees.

Table 2. Average sound seeds per cone and germination percentages for seeds produced during year +2, and tree heights of slash pines used in the study.

Treatment	Sound seeds	Germination ^x	Height	
	per cone ^x		Yr. -1	+3
	Number	Percent	--Feet--	
Banding	14	98	56	63
Spring girdling	11	97	58	64
Summer girdling	18	90	58	64
Untreated control	10	97	57	64
Average	13	96	57	64

^xValues based on data from 10 cones per tree.

Germination was high, averaging 96 percent for all treatments, and was not significantly affected by treatments (Table 2). Summer girdling resulted in the lowest germination, but this was largely due to seeds from one tree that germinated only 42 percent.

Mortality and Growth

None of the slash pines died; however, by year +2, half of the girdled longleaf trees were dead (there were 10 trees per treatment). Longleaf pine mortality by treatment in year +3 was as follows:

Treatment	Number of dead trees
Banding	2
Spring girdling	9
Summer girdling	8
Untreated control	0
Total	19

The treatments had little effect on height growth, which averaged 6 to 7 feet for all over the 3 1/2 year period. (Because of swelling above the injuries, d.b.h. measurements were not valid.)

DISCUSSION

Mortality of study trees largely precludes any realistic appraisal of the effects of the treatments on longleaf pine cone production. Only banded and control trees survived sufficiently to measure cone yields. There were no statistical differences between these two treatments and overall production was low.

Banding and girdling significantly increased slash pine cone production only in year +2--27 to 30 months after the treatments were applied. These results support Hoche's (1962) conclusions that injury increases yields of the first cone crop originating after the treatments are applied but have little effect on subsequent crops.

According to Hoekstra (1960), only 45 percent of slash pine cones on a tree are visible through binoculars from a single location. When this correction is applied to cone counts obtained from slash pine trees in year +2, the cone production per tree averaged 47 and 109 for control and injury treatments, respectively. Even with stimulation, this production is low and meaningless for seed production.

The results of this study confirm earlier tests that indicated that mechanical injury can stimulate flower and cone production in southern pines (Bilan 1960, Grano 1960, White and Wright 1987, Wheeler and Bramlett 1991). However, the results also indicate that caution must be used with these techniques. First, the magnitude of the response, although statistically significant, may not justify the risk of the treatment. Second, species seem to differ in their susceptibility to these types of injury. Longleaf pine, in particular, seems sensitive to injury. Third, girdling technique may be important. In this study the girdles were about 1-inch across, but in the tests of Wheeler and Bramlett (1991) the cuts were made with a saw and were only about 1/4-inch wide. Also, wire has been used to stimulate male flowering by banding branches (White and Wright 1987).

In general, crown release, fertilization, and irrigation have proved more reliable in the past than injury in stimulating flower and cone production of southern pines because response is more predictable and the treatments less harmful to the trees. Injury to stimulate cone production of slash and longleaf pines should be used only in special cases where some higher level of risk is acceptable.

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OPTIMUM FERTILIZER RATES FOR LOBLOLLY PINE SEED ORCHARDS

R.C. Schmidtling¹

Abstract.-- Fertilizers were applied annually for 7 years to individual ramets in a loblolly pine (*Pinus taeda* L.) seed orchard at rates ranging from 0 to 400 lbs N/acre. Treatments also included splitting the applications into spring and summer segments, and fertilizing every other year. The optimum fertilizer rate for flowering and seed production was 200 lbs N/acre/year, applied every year in mid- to late summer. Including phosphorous in the fertilizer regime appears to be desirable, whether it is applied separately in the spring or with a summer N application. The benefit of using foliar analysis to make fertilizer recommendations depends on the accuracy of the analysis used. Using heavy fertilizer rates had little effect on the concentration of foliar micronutrients.

Keywords: *Pinus taeda* L., seed orchard, cone production.

INTRODUCTION

There are many reports in the literature indicating that fertilization enhances flowering and seed production in southern pines (Schmidtling 1974). Nitrogen (N) is the acknowledged critical nutrient in flower stimulation (Sprague et al. 1978) and is the element most often used in routine seed orchard management. Fertilizers have been applied to many orchards at high rates for a number of years, but long-term effects of high fertilizer rates on cone and seed production are not known.

Shoulders (1968) reported on a long-term fertilizer rate experiment in slash (*Pinus elliottii* Engelm. var. *elliottii*) and longleaf (*P. palustris* Mill.) pine stands. He found the best flowering and cone production response with his highest rate of 150 lbs N/acre. Orchard fertilization rates have ranged from 100 to 400 lbs N/acre/year, so Shoulders' high rate of 150 lbs is on the low side compared to current orchard practice.

At moderate rates, fertilizers can improve cone and seed yields per flower as well as increase flowering (Sprague et al. 1978). Studies with longleaf pine, however, indicate that high nutrient levels may cause an increase in conelet abortion (McCall and Kellison 1981). There is other evidence that longleaf pine cannot tolerate nutrient rates as high as those that are optimum for slash and loblolly (*P. taeda* L.) pines (Schmidtling 1987).

Fertilization rates and formulations are sometimes based on soil analysis (Sprague et al. 1978), which is marginally satisfactory for nutrients other than

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nitrogen. The availability of nutrients in soils depends on more than their concentration; soil analysis is usually not done for nitrogen because there is no reliable test for available nitrogen.

Webster (1974) recommended 180 to 200 lbs N/acre/year for loblolly pine seed orchards, although the results of his nitrogen-phosphorous rate study were inconclusive. Current fertilizer rate recommendations are based on these limited research results, some preliminary results of the present study, and intuition (Jett 1986).

Foliar analysis is the logical choice for diagnosing mineral requirements for pine seed orchards (Jett 1987). Minimum standards for foliar nutrient for some nutrients have been established for forest stands of some southern pines (Leaf 1968, Pritchett 1968, Wells 1968), but little has been published on foliar standards for seed production in orchards. Webster (1974) noted an increase in foliar N and a decrease in foliar P with increasing N fertilizer rates but did not explore the use of foliar analysis for determining optimum rates.

The following are the objectives of this experiment:

- (1) To determine the long-term effects of varying rates of fertilizers on cone production,
- (2) To examine the relationship between foliar levels of macro- and micro-nutrients to explore the feasibility of foliar analysis for prescribing fertilizer treatments,
- (3) To quantify the clone X treatment interactions in (1) and (2) above. Beers (1974) proposed fertilization on a clonal "prescription" basis to maximize fertilizer efficiency. One important objective of this experiment will be to determine the magnitude of interactions in both the short and long term,
- (4) To examine the feasibility of alternative methods of application; specifically, single versus split application and annual versus biennial application.

MATERIALS AND METHODS

The study was established in the Alabama seed source at the Erambert Seed Orchard in south Mississippi. As in most production seed orchards, the makeup of orchard blocks varies considerably in age of ramets and clonal composition. Because the broad-sense heritability of flowering traits is so high, it is very important that the clonal composition of any flowering experiment be carefully balanced (Schmidtling 1974). Experiments in which whole orchard blocks are treated are often difficult to interpret because of high error rates; in this experiment, individual ramets were treated.

Thirteen clones were chosen from among the 50 available based on an inventory by clone and year of grafting. The ramets were 15 or 16 years from grafting when the experiment was initiated (age was confounded with clone, i.e., all ramets of a given clone were the same age). The ramets averaged 10 inches d.b.h. Original spacing in the orchard was 15 X 30 feet. Soils in the orchard are primarily well-drained sandy loams.

Table 1. Fertilizer rates per acre for the seven treatments of the study.

<u>Treatment</u>	<u>Nitrogen</u>	P_2O_5 (P) ^a	K_2O (K) ^a	<u>Timing</u>
- - - - Pounds/acre ^b - - - -				
1 (Control)	0	0	0	
2	100	28 (12)	28 (23)	July
3	200	56 (24)	56 (46)	July
4	300	84 (37)	84 (70)	July
5	400	112 (49)	112 (93)	July
6	400	112 (49)	112 (93)	July - Alternate years
7	56 <u>144</u> 200	56 (24) 0	56 (46) 0	April - Split July Applications

^a Elemental rates for phosphorous and potassium shown in parenthesis.

^b Spacing in the orchard is 15 X 30 ft. Fertilizer rates per acre are calculated based on 450 ft² growing space per ramet. 100 lbs/acre = 92 kg/ha.

Beginning in summer of 1982, seven treatments were applied yearly to three ramets each of the 13 clones in a factorial design (table 1). The first five treatments consist of increasing rates of N from 0 to 400 lbs/acre. The sixth treatment was included to determine whether 400 lbs/acre every other year is as effective as 200 lbs/acre every year. The seventh treatment tested the efficacy of the common practice of splitting the applications, i.e., applying a balanced fertilizer in early spring to stimulate growth and nitrogen in the summer to stimulate flowering. The overall rate is the same as the 200 lb/acre rate. A total of 273 ramets were included in the experiment.

Experience has shown that N is most often the important nutrient for flowering response (Schmidtling 1974). Severe P or potassium (K) deficiency may inhibit flowering, however, so fertilizers for most of the treatments consisted of a 50/50 mix of ammonium nitrate and 13-13-13 NPK. Fertilizers were broadcast within the "drip line" of the individual ramets at times determined to be optimum for flowering response (Schmidtling 1983) (Table 1).

From 1983 through 1986, male and female strobili were counted in the spring and cones in the fall. Foliar analysis was done on a subset of the experiment in September of 1983, 1984, 1985, 1986, and 1989. Two ramets each from the first five treatments for 10 of the 13 clones (a total of 100 trees) were analyzed for nitrogen, phosphorous, potassium, calcium (Ca), magnesium (Mg), iron (Fe), boron (B), manganese (Mn), and copper (Cu). Foliar samples consisted of most recently-formed needles from at least three branch tips from the upper crown. The foliar samples were collected in late September, just after probable formation of female strobili initials (Schmidtling 1975). Five cones per tree were collected from

this subsample in the fall of 1983 and 1984 to assess the effects of fertilization on seed yields.

SAS (1985) General Linear Model (GLM) procedure was used to test differences among treatment means in the analysis of variance for the completely random, single-tree plot design. Regression was used to test relationships between flowering (dependent variables) and foliar nutrients (independent variables). Probabilities of less than 0.05 for no difference were considered significant.

RESULTS AND DISCUSSION

As expected, the clonal effects for both male and female flowering in the analysis of the results of the experiment were always large and significant. Treatment effects were also significant for female flowering over the duration of the experiment and for male flowering the first two years (Figure 1). Clone X treatment interactions were not significant.

Fertilizer Rate

In the first 2 years after the study began, 1983 and 1984, it was obvious that the 200 lb rate was optimum for both male and female flowering (Figure 1). In 1983, controls averaged just over 160 strobili per ramet. Fertilizing with 100 lbs/acre N increased flowering to just over 180 strobili; fertilizing with 200 lbs/acre increased flowering to over 200 strobili per ramet. Higher rates did not further increase flowering. The response in number of male strobili per ramet followed a pattern similar to that of female strobili.

The pattern of flowering response in 1984 was similar to 1983, although flowering was greater in 1984 (Figure 1). The flowering response pattern changed somewhat in 1985 and 1986, when it appeared that the rates higher than 200 lbs/acre, especially the 400-lb rate, produced better results. Rather than indicating a fundamental change in the flowering response, this change is probably a result of the field design. Most of the feeder roots of the trees occur under the drip line of the crown. The fertilizers were applied with this in mind. Some roots invariably extend beyond this area and root growth occurs in response to a nutrient gradient. The most striking effect of nitrogen fertilization in agronomic crops is increased growth of roots, which brings the plant into contact with a greater quantity of nutrients (Grunes 1959). In slash pines, nitrogen fertilization has increased root growth (Schultz 1969). In this experiment, roots from adjacent, unfertilized trees probably have extended and proliferated into the area beneath the fertilized trees, absorbing nutrients and giving the appearance of a drop in rate effectiveness for the study trees.

The original study plan called for subsoiling between the ramets to minimize this effect, but the orchard manager was reluctant to do so because of the risk of increased root diseases (Webb and Alexander 1982, 1983). Such risks appear to be exaggerated (Schmidtling 1986), and subsoiling has become a common practice in seed orchards (Jett 1987).

Application Method

Applying 400 lbs/acre every other year appears to be much less effective than applying 200 lbs/acre every year. In 1983 (Figure 1), when the biennial 400-lb rate was applied, response was the same as with the regular 400 lb rate

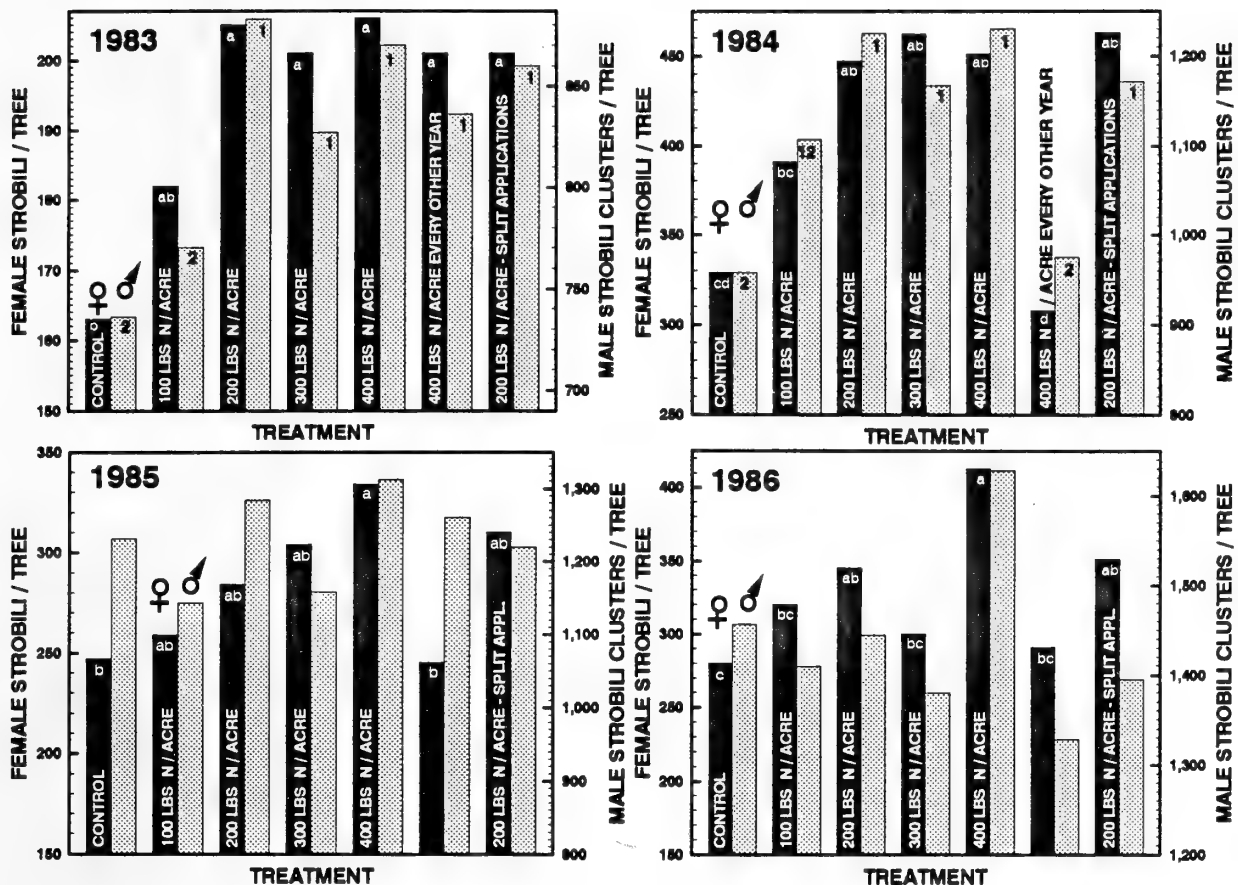


Figure 1. Male and female flowering in response to several rates and application methods of fertilizers. Bars topped by the same letter (female flowering) or number (male flowering) do not differ significantly from one another according to Duncan's multiple range test (0.05 level of significance).

and did not differ from the response with the 200 lb rate. In 1984 there appeared to be no carryover at all, and the flowering rate in the biennial treatment dropped to that of the controls. This treatment was dropped after 1984, and these trees responded similarly to the controls thereafter.

There also appeared to be no advantage to splitting the application, i.e., applying a balanced fertilizer in early spring to stimulate overall vigor, followed by applying nitrogen in summer to stimulate flowering. In all 4 years applying all the fertilizer in summer (the 200-lb treatment) was equivalent to splitting the applications (Figure 1).

Cone and Seed Yields

The treatments had no significant effect on the proportion of female strobili which were harvested as cones in any year. There were, however, small but significant effects of treatment on the yield of total numbers of seed per cone (Figure 2). In 1983, the highest rate of fertilizer appeared to reduce the total number of seed per cone from 110 in the controls to 90 seed per cone at the 400-

lb treatment. Differences among treatments in full seed per cone were not statistically significant.

The differences in total seed per cone in 1984, although statistically significant, did not agree with the pattern of differences in 1983 (Figure 2). Seed yield from trees fertilized with 200 lbs/acre was best, while yield from the trees receiving the 100-lb and 400-lb rate was poorest. Considering the results in total seed for both years and the number of full seed per cone, the 200 lb/acre rate appears to be best both for flowering and seed yield.

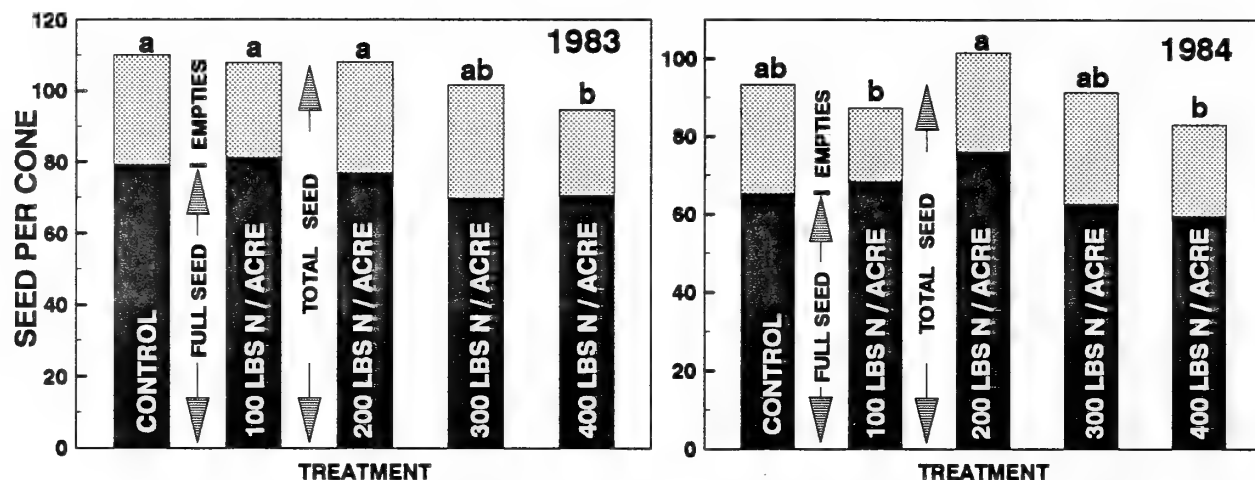


Figure 2. Seed yield in response to several rates of fertilizers. Bars topped by the same letter do not differ significantly from one another in total numbers of seed per cone according to Duncan's multiple range test (0.05 level of significance).

Foliar Nutrients

Analysis of foliar nutrients showed that clonal effects for most nutrients were significant most years. There was no relationship between flowering and foliar nutrients on a clonal basis, however. The strength of the clonal component underscores the need to consider this source of variation when sampling.

The only consistent treatment differences in foliar nutrients was for nitrogen (Figure 3a). The concentration of N generally followed the order of treatment rates, i.e., N concentration was highest in foliage from trees given the highest rates of fertilizer and lowest in foliage from the controls. There was a large overall drop in N concentration between the 1985 and the 1989 samplings (fertilizer treatments were continued during this time, although no measurements were taken). Even so, treatment effects were statistically significant and in the correct order. This may represent a change in the way the analyses were done at the commercial laboratory, since sampling methods and timing were identical to previous years.

In the earlier samplings, there appeared to be a decrease in phosphorus concentration with higher fertilizer rates, even though P was included in the

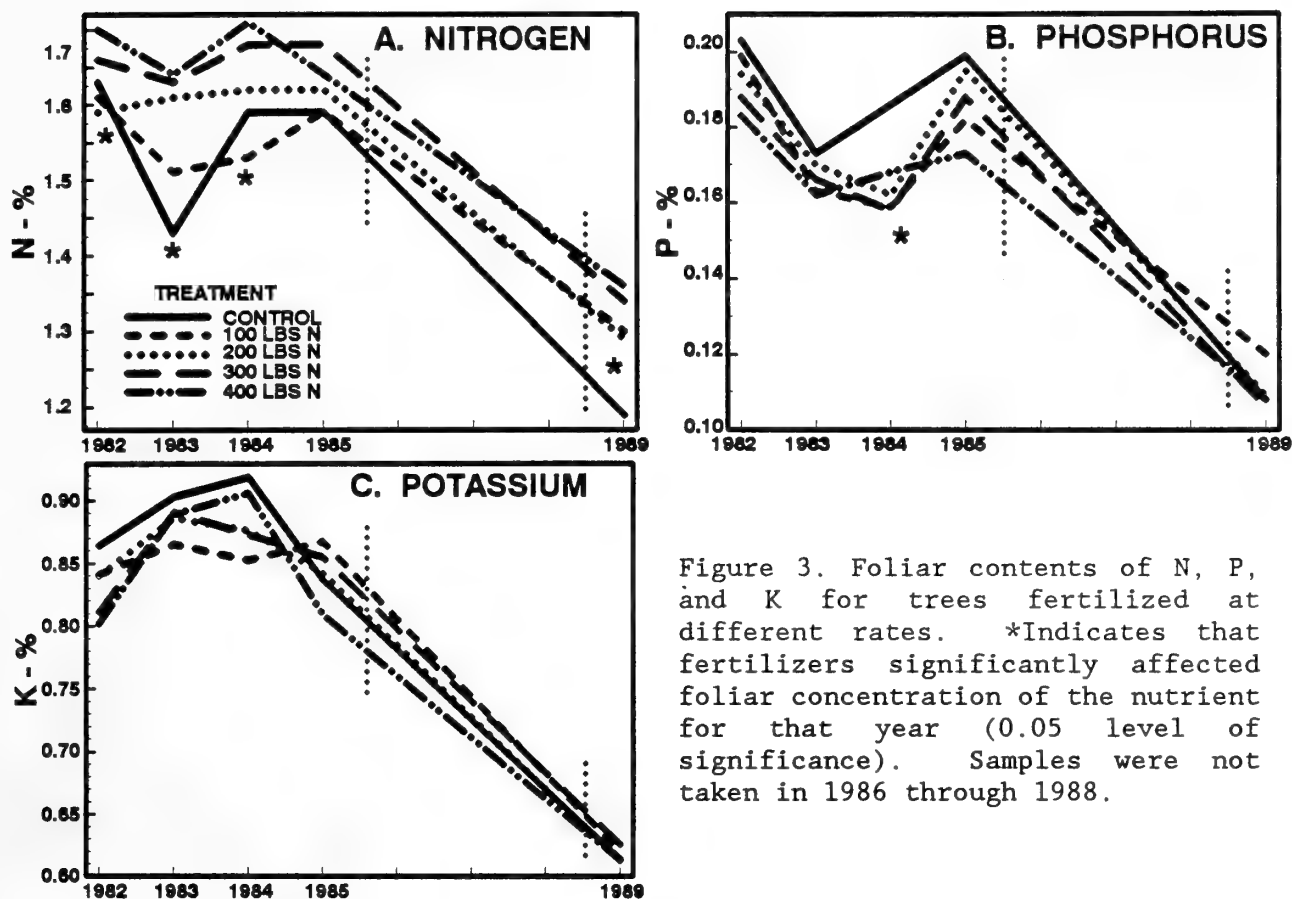


Figure 3. Foliar contents of N, P, and K for trees fertilized at different rates. *Indicates that fertilizers significantly affected foliar concentration of the nutrient for that year (0.05 level of significance). Samples were not taken in 1986 through 1988.

fertilizer mix (Table 1). A similar response was observed by Webster (1974). The difference was statistically significant in 1984 (Figure 3b). This difference was not evident in the final sampling in 1989.

There were no significant trends in the concentration of the other macro- and micronutrients over time. A decrease in micronutrients due to heavy nitrogen fertilization which was observed in *Pinus radiata* (D. Don) growing on deep sand (Woods 1983) did not occur in this experiment.

Flowering was not significantly related to concentrations of macro- or micronutrients in simple or multiple regressions. Ratios of nutrients, especially Ca/P and Ca/K were related to flowering in one report (Wilcox et al. 1991) but no significant relationship was found in the present study. The concentration of foliar N at the treatment level is related to flowering in a very general way (compare Figure 3a and Figure 1), but there was no relationship on an individual tree basis.

CONCLUSIONS AND RECOMMENDATIONS

The optimum fertilization rate for flowering and seed production in loblolly pine seed orchards appears to be 200 lbs N/acre/year, identical to the optimum rate determined for growth of loblolly pine in plantations (Ballard 1981). For best results, the fertilizer should be applied every year, in mid- to late

summer. Although the experiment did not test the effects of phosphorous application separately, the foliar analysis indicates that including phosphorous with nitrogen in the fertilizer regime appears to be desirable.

The utility of using foliar analysis to make fertilizer recommendations may depend heavily on the accuracy of the analysis used. It does seem apparent, however, that heavy fertilizer rates had little effect on the concentration of foliar micronutrients in the soils of the Erambert Seed Orchard.

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COMPARISON OF GROUND AND AERIAL APPLICATION SPRAY DRIFT
ON THE UNION CAMP CORPORATION SOUTHERN STATES LOBLOLLY PINE SEED
ORCHARD IN CLAXTON, GA

AUGUST 20-23, 1991

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Abstract.--Ground application of a spray solution containing Rhodamine WT and water applied with a FMC 757 Speed Sprayer resulted in less drift than did the application from a Cessna Ag Truck fixed wing aircraft. Spray drift was at detectable levels in small amounts to 1000 feet for the aircraft and 250 feet for the ground sprayer directly downwind of the spray block .

Keywords: Loblolly pine, Pinus taeda L, Rhodamine WT, Bullseye, Cessna Ag Truck, drift.

INTRODUCTION

The production of seed is vital to seed orchard managers and the limiting factor reducing production is damage from a variety of seed and cone insects. Various control strategies have been developed in the past to address the damage from insect pests. Presently, most orchard managers use repeated applications of a liquid insecticide applied either by ground sprayer or aircraft. The insecticide most commonly used is azinphos-methy (Guthion) applied via aircraft. In the past two years many orchard managers have switched to Foray 48B, a Bacillus thuringiensis (Bt) formulation.

The USDA Forest Service has focused on improving pesticide application deposition while minimizing off-site spray drift. All groups concerned with the application of pesticides to southern pine seed orchards recognize the importance of minimizing pesticide drift from the orchard to adjoining land or water areas. In 1980 Jack Barry (Barry et al. 1980, and Barry et al. 1982) first determined the feasibility of aially applying pesticides to southern pine seed orchards on the State of Florida, Withlacoochee Seed Orchard near Brooksville, FL. Drift in this study was confined within 133 meters downwind of the spray source.

In the Withlacoochee Trials both a Hughes 500C and a Stearman aircraft were used. The volume median diameter (VMD) of the five gallon per acre spray was 525 μm for the Hughes and 524 μm

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for the Stearman. The ratio of deposition 15 meters downwind from the orchard edge to within-orchard deposition ranged from 0.06 to 0.49 (J. W. Barry et al. 1983). Wind speed ranged up to 2.8

meters per second. In an additional test over open field conditions, spray deposit ratios at 133 meters were 0.002 with deposits of 1 oz/acre. Wind ranged from 2.7 to 5.8 meters per second. From this report Barry concluded that drift deposits 60 meters downwind of seed orchards in flat terrain is about 8 percent of the amount deposited in the treatment area and can be expected to 100 meters. They also concluded that large amounts of spray are deposited with a 15 meter zone surrounding orchards.

In 1991 a study was undertaken to compare ground and aerial application on the Union Camp Southern States loblolly pine seed orchard. This study evaluated deposition within the tree canopy, forest floor and downwind (Barber and Mangini 1993).

MATERIALS AND METHODS

Scope

This was a cooperative project between the USDA Forest Service and Union Camp Corporation conducted August 20-22, 1991. There were two trials each morning that compared ground and aerial application.

Site Description

The orchard is located 4.5 mi south and west of Claxton, Ga. The orchard has tree spacing of 22 x 22 ft (6.7m) . The test site was the 8.9 acre Alabama rust resistant loblolly pine, Pinus taeda L., area of the orchard.

Meteorological Measurement

Meteorological conditions were measured with: 1) a Handar 540A on a 22 ft (6.7 m) tower located near the center of the spray block, 2) another Handar 540A on a 55 foot (16.8 m) tower located 600 ft (182.9 m) northeast of the spray block and 3) a Forest Technology System F11 on a 6 ft (1.8 m) tower located 300 ft (91.4 m) east of the spray block in an open field.

Application

The aircraft sprayed a mixture of Bullseye dye and water and the ground sprayer applied a mixture of Rhodamine WT dye and water. The aircraft application was 1.19 gal/acre (4.5 l) while the ground sprayer application rate was 2.88 gal/acre (10.9 l) for days 1 and 2 and 5.76 gal/acre (21.8 l) for day 3. The aircraft was a Cessna Ag truck Model 185 flying at 110 mi/h (177 km). The ground sprayer was a FMC 757 Speed Sprayer traveling at 2.5 mi/h (4.0 km).

Drift Sampling

During each spray application, drift lines were established beginning 50 ft (15.3 m) within the orchard and extending out from the spray block downwind in 50 ft increments. Drift line samplers were Kromekote cards 4 x 5 in (10.2 x 12.7 cm).

Stain Deposit Measurement

Stains on Kromekote cards were measured by placing each card under a dissecting microscope fitted with a graduated measuring reticule. On each card a minimum of 50 stains were counted and the area observed did not exceed 16 cm². Spray stain numbers and sizes were analyzed using the Automated Spot Counting and Sizing program (ASCAS, Continuum Dynamics) to convert drop counts into spray volumes. The spray volume and numbers of drops/cm² were adjusted for the ground sprayer application rate to equal the aircraft application rate. When equivalent deposition is used it is noted.

RESULTS

Characterization

Volume median diameter (VMD) for the aircraft was 196, 136, and 127 microns for days 1, 2, and 3 respectively. The effective swath width (eight drop/cm² minimum deposition) for days 1, 2, and 3 were calculated to be 90, 110, and 100 ft respectively. No swath width was determined for the ground sprayer, however, the VMD for day 3 was 202 microns.

Meteorological Conditions

On day 1 maximum wind ranged from 5.5 mi/h to 7.2 mi/h and was out of the west-southwest with temperatures in the lower eighties with relative humidities from 69.5 to 89 percent. During day 2 the wind was from the west at less than 2 mi/h. On day 3 wind was 2.6 mi/h from the north-northwest. The temperature ranged from 72.4 F° to 82.3 F° with the relative humidity from 68.7 to 90 percent.

Spray Drift

Day 1

Deposition on the ground within the orchard and at the orchard edge for both the aircraft and ground sprayer ranged from 44.9 to 46.5 fl oz/acre (tables 1 and 2). Aircraft deposition dropped off rapidly with increased distance from the orchard edge and at 150 feet only 6.26 fl oz/acre were detected. Here a ratio of only 0.2:1 was found as compared to within the orchard (table 3). This is 20 percent deposition compared to within the orchard. At 300 feet 0.15 fl oz/acre were detected or 0.1 percent of the deposition within the orchard. Ground sprayer deposition 150 feet

outside the orchard was 3.95 fl oz/acre or 11 percent of within orchard deposition. At 300 feet deposition was 0.1 fl oz/acre or 0.1 percent of within orchard deposition.

Day 2

Aircraft deposition directly downwind of the spray block at 150 feet from the orchard edge was 6.89 fl oz/acre or 18 percent of the deposition within the orchard. Ground sprayer deposition however was 0.38 fl oz/acre or 3 percent of the within orchard deposition. All cards beyond 150 feet received spray deposition but were wet and unreadable.

Day 3

Spray deposition from the aircraft at 150 feet from the orchard edge was 25.08 fl oz/acre or 111 percent of that found within the orchard. At 300 feet the deposition fell to 4.19 fl oz/acre and at 400 feet was 0.78. This represented 5 and 1 percent respectively of the deposition within the orchard. Aircraft deposition was 0.02 fl oz/acre at 1000 feet. Ground sprayer deposition at 150 ft was 0.17 fl oz/acre and 0.09 fl oz/acre at 250 ft with no further deposition detected to 1000 feet.

Table 1 - Ground spray deposition on drift line samplers - Claxton Spray Trials, 1991

Drift line 1

	Day 1	Day 2	Day 3
Card	Fluid	Fluid	Fluid
position	ounces/acre	ounces/acre	ounces/acre
-50.00	44.90	6.19	31.04
0.00	29.75	20.18	17.33
50.00	12.18	56.10	26.55
100.00	21.14	4.20	1.97
150.00	3.95	0.38	0.17
200.00	0.73	wc	0.17
250.00	1.10	wc	0.09
300.00	0.01	wc	0.00

Table 2 - Aircraft spray deposition on drift line samplers - Claxton Spray Trials, 1991.

Drift line 1

	Day 1	Day 2	Day 3
Card	Fluid	Fluid	Fluid
position	ounces/acre	ounces/acre	ounces/acre
-50.00	17.22	76.56	43.72
0.00	46.51	0.98	1.41
50.00	31.21	36.98	31.09
100.00	17.27	48.91	33.81
150.00	6.28	6.89	25.08
200.00	2.63	wc	11.57
250.00	5.59	wc	4.19

300.00	0.15	we	4.19
350.00	NA	NA	1.07
400.00	NA	NA	0.78
450.00	NA	NA	0.30
500.00	NA	NA	0.07
550.00	NA	NA	0.20
600.00	NA	NA	0.01
650.00	NA	NA	0.11
700.00	NA	NA	0.17
750.00	NA	NA	0.00
800.00	NA	NA	0.16
850.00	NA	NA	0.01
900.00	NA	NA	0.01
950.00	NA	NA	0.01
1,000.00	NA	NA	0.02

Table 3 - Drift deposit ratios from aerial and ground tank mixes comparing deposition within the orchard to drift sites outside the orchard - Claxton Spray Trials, 1991

Drift line 1

Card position	<u>Day 1</u>		<u>Day 2</u>		<u>Day 3</u>	
	Ground sprayer	Aircraft	Ground sprayer	Aircraft	Ground sprayer	Aircraft
50.00	0.33	0.98	4.25	0.95	1.10	1.38
100.00	0.58	0.54	0.32	1.26	0.08	1.50
150.00	0.11	0.20	0.03	0.18	0.01	1.11
200.00	0.02	0.08	we	we	0.01	0.51
250.00	0.03	0.18	we	we	0.04	0.19
300.00	0.0002	0.001	we	we	0.00	0.05
350.00	NA	NA	NA	NA	0.00	0.03
400.00	NA	NA	NA	NA	0.00	0.01
450.00	NA	NA	NA	NA	0.00	0.003
500.00	NA	NA	NA	NA	0.00	0.01
550.00	NA	NA	NA	NA	0.00	0.0004
600.00	NA	NA	NA	NA	0.00	0.005
650.00	NA	NA	NA	NA	0.00	0.01
700.00	NA	NA	NA	NA	0.00	0.00
750.00	NA	NA	NA	NA	0.00	0.01
800.00	NA	NA	NA	NA	0.00	0.00
850.00	NA	NA	NA	NA	0.00	0.0004
900.00	NA	NA	NA	NA	0.00	0.0004
950.00	NA	NA	NA	NA	0.00	0.0004
1,000.00	NA	NA	NA	NA	0.00	0.0009

DISCUSSION

Spray drift downwind and off-site from a seed orchard spray block appears to be more likely with aerial application compared with ground based sprays where small droplet sizes are used and when comparing equal amounts of spray solution per acre. Under normal orchard aerial spray conditions using the pesticide Guthion, the VMD is usually greater than 350 microns (Barber and Fatzinger 1987). In this test the VMD ranged between 127 and 196 microns. Many orchard managers are currently using Foray 48B applied at 1 gal/acre with VMD's similar to this study. In these cases drift beyond 300 feet from the orchard edge may be expected. This drift may not pose a significant problem on orchards as most orchards have a pollen buffer zone around the orchard. All Federal orchards have a 400 ft buffer to minimize pollen from entering the orchard. Potential drift from the orchard would be deposited on this buffer strip. The resulting off-site deposition in this study would have been less than 1 fl oz/acre beyond 400 feet of the orchard edge. Using larger droplet sizes would also reduce drift from the orchard.

The ground sprayer solution in this study did not drift far from the orchard but remained on site. On day three, deposits downwind of the orchard were detected to only 250 feet with none found after that point. This compares to aircraft deposition to 1000 feet. Ground sprayers however apply from 10 to 100 times more volume per acre than aircraft under orchard conditions and the resulting spray would result in considerable deposition within the orchard. Unfortunately, this deposition is found on the ground and on the lower branches (Barber and Mangini 1993) while the cone crop is found on the upper half of the crown. Previous work has shown more drift downwind when using ground sprayers (Ware et al. 1969). Possibly this is because the previous work was in open fields. This study was in a mature seed orchard. Under these conditions most of the spray material was deposited on the bottom branches and did not reach the upper crowns in quantities to move above the trees where drift could occur. In comparison the aircraft released its solution 15 to 20 ft above the trees.

CONCLUSIONS

Off-site aerial spray drift using droplet sizes less than 200 microns may result in spray deposition to 1000 feet, however, the majority of the off-site deposition was contained to within 300 ft of the orchard edge. This deposition would fall within a pollen management buffer strip which is located on most orchards. Because most orchard managers apply Guthion at 10 gallons per acre with a VMD of 350 microns or greater, less drift would be expected under these operational parameters.

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Disclaimer

The use of trade, firm, or corporation names is for the information and convenience of the reader. Such use does not constitute an official evaluation, conclusion, recommendation, endorsement, or approval of any product or service to the exclusion of others which may be suitable.

Caution: Pesticides can be injurious to humans, domestic animals, desirable plants, and fish or other wildlife if they are not handled or applied properly. Use all pesticides selectively and carefully. Follow recommended practices for the disposal of surplus pesticides and pesticide containers.

SESSION 2

Hardwood Genetics and Propagation

GENETIC VARIATION FOR ROOTING, GROWTH, FROST HARDINESS, AND WOOD, FIBER, AND PULPING PROPERTIES IN FLORIDA-GROWN EUCALYPTUS AMPLIFOLIA

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Abstract. Variability in clonal rooting, clonal and seedling growth and frost-tolerance, and wood properties was noted in a 1st-generation E. amplifolia genetic base population in Florida. Rooting success averaged only 40% but varied considerably among 84 cloning candidates. Their growth in clonal tests also varied, while many new introductions grew as well as the best of previous accessions in addition to providing comparable or better frost tolerance. Variability among and within families was significant for frost tolerance. Artificial freeze testing procedures hold promise for screening accessions and identifying frost-tolerant individuals. Wood specific gravity (SG) and moisture content (MC), pulp yield and viscosity, and other important fiber and paper characteristics varied among- and within-provenances. Compared to sweetgum, E. amplifolia was lower in SG, pulp yield, and sheet strength but less consumptive of energy and chemicals for bleaching to similar brightness; and higher in smoothness and opacity for writing grade papers.

Keywords: Provenances, progenies, clones, Australia, pulp and paper.

INTRODUCTION

Eucalyptus amplifolia Naud. has grown well on good sites in peninsular Florida (Rockwood et al. 1991) but needs further evaluation. Provenance, progeny, and tree variability was significant for growth and frost-tolerance, and E. amplifolia progenies were more frost-resilient than four other Eucalyptus species. The rooting abilities of cloning candidates varied. Relatively little is known about its wood properties in Australia (Dadswell 1972) or elsewhere. This paper reports recent results on the following aspects critical to its use: 1) clonal rooting and testing, 2) growth in an expanded 1st-generation genetic base population, 3) artificial freeze screening, and 4) basic wood, fiber, and pulping properties.

MATERIALS AND METHODS

Rooting Study. Over 5,700 coppice stem cuttings of 84 selections from a previous base population (Rockwood et al. 1991) were rooted in May 1992 using a commercial rooting hormone, after soaking the cuttings in a fungicide. After 40 days in mist, rooting percentages were determined. Ramets were outplanted in studies SRWC-47, -48, and -50 (Table 1) and seed orchard CT74 near LaBelle in August.

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Table 1. Means and ranges for height (in m) of 31 *E. amplifolia* progenies and up to 63 clones in three field studies established in 1992.

Field studies established in 1992:										
Entry		SRWC-47			SRWC-48			SRWC-50		
		No.	Mean	Range	No.	Mean	Range	No.	Mean	Range
			11-mo Height			13-mo Height			6-mo Height	
Progenies: Tested		9	0.9	0.6-1.3*	9	3.0	1.5-4.0	9	0.7	0.4-1.0**
New		22	0.9	0.7-1.3*	22	3.0	2.0-4.0	22	0.7	0.5-1.0**
			6-mo Height			9-mo Height			2-mo Height	
Clones:	Set 1	2	0.3	0.2-0.5	21	1.0	0.5-1.7	21	0.4	0.2-0.5
	Set 2				21	0.9	0.3-1.7	24	0.3	0.2-0.5
	Set 3				14	1.3	0.8-2.0	18	0.3	0.1-0.6**

* and ** - Progeny/Clone variability significant at the 5% and 1% levels, respectively

Growth Studies. In April 1992, three studies of 31 progenies, 22 new introductions from frost-frequent regions of northern New South Wales (NSW), Australia, plus the nine best progenies in previous studies (Rockwood et al. 1991), were established in Florida (Table 1). Study SRWC-47 was planted near Zephyrhills at 2.4 x 1.5 m spacing in a randomized complete block design with three replications of 5-, 6-, and 7-tree row plots, respectively, due to space/slope constraints. Study SRWC-48 at Gainesville used a 2 x 1 m spacing with 8-tree row plots within three replications of a randomized complete block design. Study SRWC-50 was installed near Flemington at 3 x 2 m spacing with three replications of 6-tree row plots. Survival, height, and DBH were measured periodically through May 1993.

Freeze Studies. In Freeze Study 1, five seedlot groups (Table 2) were screened by the CSIRO Division of Forestry. Groups 1 and 2 were five frost "resistant" and five frost "sensitive" seedlots, respectively (based on field tests in Florida), from a broad region in northern NSW. Group 3 was five untested seedlots originating east of Guyra, NSW. The *E. grandis* Hill ex Maid. and *E. regnans* F. Muell. groups provided baselines for assessing relative species tolerance: Group 4 - two frost resilient *E. grandis* seedlots from a 4th-generation seed orchard in Florida (Rockwood et al. 1989), Group 5 - included one standard *E. regnans* seedlot widely used in previous tests (Raymond et al. 1992a). Hardening of some 128 trees was accomplished by 38 days under shade cloth at night temperatures of 6°C or less followed by 21 days of 3°C or less night temperatures without cover. For hardened trees, liquid cold bath temperatures were -5.5, -7.0, and -8.5°C and for unhardened seedlings -1.0, -2.3, and -3.6°C. Frost tolerance was determined using the relative conductivity (RC) technique of Raymond et al. (1986, 1992a).

In Freeze Study 2, four *E. amplifolia* families, including three in Freeze Study 1 (Table 2), were used to assess a cold bath testing procedure developed for citrus in Florida. Eleven vigorous 2-month-old seedlings of each progeny were subjected to 15 hour nights at 4°C for hardening. After one month, progeny comparisons were conducted using 6mm disks from two leaves, i. e., one detached leaf from each of the first two fully expanded leaf pairs of each seedling. To assess variability between leaf pairs, a second leaf was taken from each leaf pair in family 4809. A second leaf in each pair for another two families was used to a) compare 6mm with 8mm disks in the case of family 4873, and b) contrast RC with visual whole leaf damage in family 5010. After exposing each leaf or disk to -5.5°C, RC was determined by procedures similar to those of Freeze Study 1.

Freeze Study 3 applied a modification of the Freeze Study 2 procedure to progenies and clones in SRWC-48. Two vigorous young leaves were collected from up to five unhardened ramets of each of 55 clones and up to 24 unhardened seedlings of each of 15 progenies (Table 2). Similar leaves were also collected from four to six hardened (21 days of 15-hour nights at 4°C) ramets of five of the 55 clones.

Table 2. Relative conductivity (RC) of various *Eucalyptus* groups in three artificial freeze studies.

Group-Family	Florida Freeze Rating (% Undamaged)	Study 1 ¹				Study 2		Study 3	
		Unhardened		Hardened		Hardened		Unhardened	
		No.	RC	No.	RC	No.	RC	No.	RC
1-"Resistant" <i>E. amplifolia</i>			.826		.857				
4809	60.0	15	.841	10	.871	11	.556	23	.735
4822	80.0	15	.746	7	.835				
4842	55.0	13	.830	5	.825	11	.459	12	.757
4859	52.3	15	.865	7	.882				
4861	64.3	12	.853	5	.856				
2-"Sensitive" <i>E. amplifolia</i>			.746		.793				
4812	11.1	15	.614	10	.726				
4825	28.6	12	.800	5	.850				
4830	10.0	15	.884	9	.865				
4831	10.5	13	.787	4	.759				
4844	10.5	15	.662	10	.782				
3-Untested <i>E. amplifolia</i>			.815		.792			187	.699
4869								11	.752
4870								13	.713
4871								14	.689
4872								14	.708
4873						11	.419	24	.711
4875								14	.682
5010		15	.812	7	.850	11	.486	20	.693
5011		14	.838	10	.717			12	.711
5012		15	.858	5	.771			11	.678
5017		11	.764	8	.847			14	.667
5018		10	.777	0	-			10	.661
5020								15	.671
5022								16	.739
4- <i>E. grandis</i>			.791		.651				
2814		10	.804	4	.641				
2817		12	.780	8	.656				
5- <i>E. regnans</i>			.615		.722				
MG98		14	.752	10	.747				
108		14	.478	4	.661				
<i>E. amplifolia</i> Cloning Candidates								157	.721

¹Results for temperatures of -2.3 and -5.5°C for unhardened and hardened trees, respectively

A 6 mm disk removed from each leaf by a paper punch was placed in a test tube containing frozen deionized distilled water. Unhardened trees were then exposed to -2.3°C; hardened trees were screened at -5.5°C. RC was determined as in the previous studies.

Wood, Fiber, and Pulping Studies. Four studies (basic wood properties, preliminary pulping assessment, comparative pulping, and comparative bleaching) were conducted using trees established at Belle Glade (Rockwood et al. 1987). Three 53-month-old trees between 8 and 14 cm in DBH per three provenances were felled in December 1989. Each tree was measured for DBH and total height before being bucked and measured for diameter at 1.5 m intervals. At each interval, a disk was removed, stored in a refrigerator, and processed for SG and MC determinations within a month. Stem sections were shipped to IPST where they were frozen until processed. Stem material between 0.5 and 2.5m above ground was manually debarked, chipped, and sized. In the preliminary Kraft process pulping assessment, the effects of amount of alkali and total H-factor on pulp yield, intrinsic viscosity, and Kappa number were assessed. The "cooks" for the comparative pulping study used a derived H-factor of 700 and an alkali charge of 13% to assess pulping yield, viscosity, Kappa number, fiber length, etc. For comparison of pulping properties, a 38-year-old sweetgum, Liquidambar styraciflua, from northwestern Georgia was also processed. In the bleaching study, samples of provenances 2-3 and 2-7, which had distinctly different characteristics in the comparative pulping study, and sweetgum were compared for brightness, viscosity, and strength. Bleaching was done in three stages: chlorine dioxide delignification, caustic extraction, and chlorine dioxide brightening. From each fully bleached sample, 30g subsamples were removed, refined in a PFI mill, formed into handsheets and brightness pads, and subjected to physical testing.

RESULTS AND DISCUSSION

Rooting Study. Rooted cutting survival in 1992 was very similar to results in previous years with different clones. The 84 cloning candidates had an average rooting of 40%, with individual clones rooting from 0 to 97%. Only seven clones, about 8% of all clones, equalled or exceeded the 80% rooting typically achieved for E. grandis in Florida (Meskimen et al. 1987). In two previous years, 10 clones rooted at 41%, and eight clones averaged 39%, with respective variation among clones ranging from 2 to 71% and 0 to 84% (Rockwood et al. 1991).

The notable similarity in rooting average and variability across years raises several concerns. A rooting rate averaging only 40% makes commercial rooting of superior E. amplifolia clones more expensive and thus less feasible, although a few clones could apparently be readily propagated by rooting. The time needed for cuttings to develop good root systems also varied greatly. Many clones, though, rooted very poorly, e. g., 32% of the 84 clones had rooting less than 20% and eight clones, including some very superior phenotypes, failed to root. Micropropagation does not appear to be a viable alternative method of vegetative propagation for E. amplifolia (Rockwood et al. 1991).

Some 32 of these 84 clones from the first E. amplifolia genetic base population (Rockwood et al. 1991) were interplanted in seed orchard CT74 in southern Florida, which typically has milder winters than northern Florida. A seedling seed orchard for E. amplifolia in northern Florida, patterned after a successful program conducted for E. grandis in southern Florida (Reddy et al. 1986), would involve much risk to developing flower crops. In CT74, these clones may by natural crossing accomplish two objectives: 1) produce E. amplifolia seedlings adapted to Florida by matings among themselves and 2) generate useful hybrids by crossing with other redgums to combine desirable aspects such as the superior frost-tolerance of E. amplifolia and the high rooting ability and site adaptability of Florida-adapted E. camaldulensis and E. tereticornis Sm.

Growth Studies. Early height growth in three field studies varied considerably among progenies, but the previously tested progenies did not grow more than the new accessions (Table 1). In each of the three studies, the average height of nine "tested" progenies was virtually the same as the average of the 22 accessions from more frost frequent areas. For both tested and new progenies, the tallest progenies were nearly twice the size of the shortest progenies. At this early stage, the broadening of the genetic base

accomplished through these new accessions seems helpful.

Clones appear to vary in early height (Table 1). The randomly formed three sets of clones in Studies SRWC-48 and -50 were similar in height after nine and two months, respectively, but clones within sets ranged widely. Should these differences be maintained, many cloning candidates will be discarded due to very poor vigor. Additional clonal deletions may be necessary because of poor stem form. Combined with further culling for frost tolerance, relatively few of the current candidates are likely to be superior clones, indicating the challenge in developing *E. amplifolia* for Florida. In developing fast-growing, freeze-resilient *E. grandis* clones for Florida, the "keep rate" was .0037% (Meskimen et al. 1987).

Because the clones were nearly five months younger than the progenies in each study, no direct growth comparisons between the two were made. However, much greater tree-to-tree uniformity is already apparent in the clones. Within-progeny variability for tree size and form for these *E. amplifolia* seedlots was relatively large and far exceeded within-clone variability.

Freeze Studies. In Freeze Study 1, temperature ($p < 0.01$) and group ($p < 0.05$) effects on RC were significant for both hardened and unhardened trees (Table 2). The most discriminating temperatures were -5.5°C for hardened trees and -2.3°C for unhardened trees. Unhardened *E. grandis* were intermediate for frost tolerance whilst *E. regnans* was the least tolerant. Hardened *E. amplifolia* seedlots were also more frost tolerant than either *E. regnans* or *E. grandis*. The ranking of *E. regnans* did not change markedly between the hardened and unhardened data indicating that the degree of hardening achieved was limited due to the relatively mild Canberra (1991) winter. With more typical winter conditions, *E. regnans* would be expected to continue to harden and eventually be able to withstand lower temperatures than the other two species. The generally greater frost tolerance of the *E. amplifolia* progenies over *E. grandis* conforms to previous species comparisons in field and artificial freeze tests in Florida (Rockwood et al. 1991).

The interaction of temperature with group was significant for the hardened trees ($p < 0.05$) but was not significant for the unhardened. Both family within group and tree within family effects were also significant ($p < 0.05$) for all groups combined. However, the interaction of temperature with family within group was not significant for hardened trees and significant for the unhardened ($p < 0.01$).

Within the three *E. amplifolia* groups over all hardened and unhardened seedlings, the effects of temperature were significant ($p < 0.01$), but group effects were not because of considerable variation in frost tolerance within each group. However, both family within group and tree within family effects were significant ($p < 0.05$) for both hardened and unhardened trees. Interactions between temperature and group were not significant. Families from all three groups were in the top three in the unhardened data and in the top five in the hardened data. Only families 5011 and 5012 differed significantly in ranking from unhardened to hardened, suggesting different responses to hardening. Family 4830 was the most frost tolerant family (unhardened) although it was from the "Sensitive" group, indicating that selection for frost tolerance should not be limited to the most tolerant provenances or groups.

Individual trees were also an important source of variation. The percentage of trees occurring in each third of the frost tolerance rankings was consistent for the hardened and unhardened material. Groups 2 and 3 had almost as many frost tolerant trees as group 1 in the top third of the rankings. Only one seedling (from group 1, family 4861 hardened) remained alive at -8.5°C .

Within Freeze Study 1 and within a Florida field study, various freeze damage measures ranked *E. amplifolia* families consistently, but families performed somewhat inconsistently across artificial and field studies. The correlation between family means for unhardened trees tested at -2.3°C and hardened trees

tested at -5.5°C in Freeze Study 1 was highly significant at 0.84. Two measures of damage (percent of trees undamaged and percent of stem killed) by a February 1989 Florida freeze (as low as -5°C for 10+ hours) also were strongly associated ($r=0.91$), with percent trees undamaged (Table 2) being slightly more discriminating among families. However, the relationship between the Freeze Study 1 unhardened means and the Florida percent undamaged was a non-significant 0.57, primarily because of the inconsistent performance of families 4822, 4825, and 4830. Seedlings of the "sensitive" Family 4830 may have given "resistant" RCs because of their non-healthy condition.

Freeze Study 2 provided useful guidelines on procedures for assessing frost tolerance in E. amplifolia progenies. Variability between leaves within a pair appeared insignificant. Adjacent fully developed pairs of leaves also varied little. Leaf disk sizes, specifically 6mm vs. 8mm diameters, did not affect RC assessments. Visual leaf damage appraisal though was not as reliable as RC determinations. In order to estimate family frost tolerance with 95% confidence with 10% precision, 10 hardened trees per family must be sampled if two disks are collected per tree. Although the RC levels in this study were much lower than in Freeze Study 1, the three families common to both studies were ranked similarly.

Genetic sources of variation significant in Freeze Study 1 were also evident in Freeze Study 3 (Table 2). Differences among families were large; the two "Resistant" families were more frost tolerant than most of the new untested accessions. Allowing for different RC levels in Freeze Studies 1 and 3, the new accessions on average are probably intermediate in frost tolerance between the Florida field tested "Resistant" and "Sensitive" families. Variability among trees in families was again significant, suggesting that individual trees offer promise for increasing frost tolerance.

Clonal variability in Freeze Study 3 was also considerable. The average tolerance of the 55 clones was less than the two "Resistant" families and more than the untested families. Individual clones were as low as .573 in RC to as high as .842, indicating that many of the trees selected in response to the February 1989 freeze were not highly freeze tolerant. When selected in August 1989, these clones were considerably larger and more frost-resistant to typical freezes in northern Florida than unselected E. amplifolia, but they had not been subjected to the extreme cold represented by the rigorous December 1989 freeze, which killed most stems to the ground. The stems of the few trees without complete stem kill were dead to at least an 8 cm diameter. All trees, however, coppiced.

Given the uncertainties of field testing new introductions and cloning candidates for frost tolerance, two-stage evaluations involving laboratory screening such as done by Harwood (1983), Eldridge et al. (1983), and Raymond et al. (1986, 1992a, 1992b) appear essential for developing frost tolerance in E. amplifolia. Freeze Study 3 defined a fast and efficient screening procedure. As in Freeze Study 2, two leaves per tree gave very similar RC values, providing further evidence that sampling one leaf per unhardened tree may be sufficient. The use of unhardened instead of hardened trees for screening is supported by results in Freeze Study 1 but is unclear based on the comparison of five unhardened and hardened clones in Freeze Study 3. In Freeze Study 3, over 650 leaf disks (325 trees) were collected and processed by three people in one day. One person completed the RC readings over another two days. Thus, more than 30 families (at 10 trees per family) could be evaluated in a three-day period.

Wood, Fiber, and Pulping Studies. Between- and within-provenance variability was important for many properties (Table 3). Provenance 2-3 had significantly higher SG than the other two provenances. Consequently, it also had lower MC. Its generally different wood and pulping properties, in contrast to provenance 2-7, served to bracket the range that could be expected in paper made from E. amplifolia.

Significant variation of wood/pulp/paper properties among trees within provenances suggests prospects to select trees with very favorable properties. Selection on the basis of wood properties, instead

Table 3. Species and provenance means for tree DBH and total height and various wood, pulp, fiber, and paper properties of 53-month-old *E. amplifolia* provenances and a 38-year-old sweetgum check.

Species - Provenance	DBH (cm)	TH (m)	Wood		Unbleached Properties				Bleached Properties			
			SG (g/cc)	MC (%)	Pulp Yield (%)	Kappa No.	Visc. (cP)	Fiber Length (mm)	Bright. (%)	Poros. ¹ (s)	Tensile Index ¹ (mN m ² /g)	Visc. (cP)
<i>E. amplifolia</i>												
2-3	10.3	12.7	0.532	109.0	46.5	20.0	55.7	0.571	88.2	5	58	22.4
2-7	11.3	12.4	0.433	148.2	45.7	19.2	46.7	0.483	86.8	50	72	39.6
2-8	11.5	13.4	0.440	151.0	46.8	19.4	48.1	0.468	-	-	-	-
Mean	11.0	12.8	0.468	136.1	46.3	19.5	50.2	0.507	-	-	-	-
Sweetgum	18.0	18.7	0.503	-	48.5	20.8	65.5	1.327	85.3	92	88	49.0

¹estimated at 300 CSF

of pulp and paper properties, may be possible. SG was negatively correlated with pulp viscosity ($r = -0.92$), sheet density, and tensile strength and positively associated with fiber length ($r = 0.81$).

Comparisons with sweetgum reveal specific advantages and disadvantages of *E. amplifolia* relative to this common native hardwood (Table 3). Overall, *E. amplifolia* had lower SG, sheet strength, and tensile index and had much shorter fibers. Sweetgum gave a higher pulp yield, but less energy and chemicals were required to bleach *E. amplifolia* to comparable brightness. Less energy was needed to reduce *E. amplifolia* to a given freeness level. Because of finer fibers and abundant parenchyma, *E. amplifolia* had a lower unrefined freeness. These finer fibers also provided a greater scattering coefficient, that for a given sheet weight and density produced more opacity. Given its high scattering coefficient and smoothness of pulp handsheets, *E. amplifolia* pulp would be a suitable component of a blended furnish used to make printing and writing grades of papers. These properties of *E. amplifolia* are similar to those of four eucalypts which can be grown in southern Florida. Franklin (1977) concluded that *E. camaldulensis*, *E. grandis*, and *E. robusta* Sm. pulp yield by the Kraft process was equal to native hardwoods, handsheet strength was less, and brightness was superior.

While *E. amplifolia* now shows more promise for good or amended sites in peninsular Florida, various reservations must be realized. True frost tolerance cannot yet be expected in rigorous northern Florida winters. Rather, fast-growing, freeze-screened progenies and clones should be considered freeze-resilient, i. e., sustain minimal freeze damage and regrow vigorously. Superior individual trees cannot be assuredly propagated vegetatively. Planting stock that combines good growth, frost tolerance, and desirable wood/pulp/paper qualities is a challenging goal requiring large-scale importation and testing.

CONCLUSIONS

Genetically improved *E. amplifolia* may be suited to fertile or enriched sites in Florida. Clonal propagation is possible through rooted cuttings, but the rooting abilities of cloning candidates are extremely variable. Genetic testing is necessary to identify vigorous progenies and clones. Excellent frost resilience may be expected, but additional frost resistance must be developed. Significant genetic variation in frost tolerance at the family and within family levels was detected in both hardened and hardened *E. amplifolia* in artificial freeze tests. Wood specific gravity and moisture content varied

among- and within-provenances. Specific gravity was negatively correlated with pulp viscosity, sheet density, and sheet strength, and positively correlated with fiber length. Compared to sweetgum, E. amplifolia was lower in wood density, fiber length, pulp yield, sheet strength, and energy and chemicals consumption for bleaching to similar brightness; and higher in smoothness and opacity for writing grade papers. Additional emphases on developing trees with growth and frost resilience by introduction and testing through field and artificial freeze screening, and clonal propagation are suggested.

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TOWARD A SINGLE NURSERY PROTOCOL FOR OAK SEEDLINGS

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ABSTRACT

After a soil fertility baseline had been determined for the Georgia Forestry Commission's (GFC) Morgan Nursery, a single nursery protocol consistently produced high quality oak seedlings. The fertility baseline developed at the Institute of Tree Root Biology (ITRB) Whitehall Experimental Nursery and adjusted for three GFC nurseries has a background target level at CA, K, P, Mg, Cu, Zn, and B of 500, 80, 80, 50, 0.3-3, 3-8 and 0.4-1.2 ppm, respectively, at time of sowing. Up to 1345 kg/ha of NH_4NO_3 are applied throughout the growing season in small increments at regularly scheduled intervals. When most seedlings are entering their second growth flush regular irrigation is stopped, and water is provided only when tensionmeters register 30 to 50 centibars. The desired seedbed density is between 54 and 57/m².

This protocol permits ready identification of competitive seedlings. Individuals with the potential to develop high numbers of first-order lateral roots (FOLR) are well established in the dominant canopy in nursery beds. Based on stem and root characteristics, approximately half of the seedlings of most species can be classified as desirable. For most species of oak, competitive ability of these seedlings after outplanting has not been determined. Specific species attributes based on their site required are obvious when seedlings are lifted and are discussed.

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INTRODUCTION

Over 30 species of oak are important components of southern forests and all of them may become important in different tree improvement programs there. Presently only 5 or 6 species are normally grown in southern forest tree nurseries and production is low. However, even with those most commonly grown, northern red oak (Quercus rubra) and white oak (Q. alba), it has been difficult to consistently produce large, high quality seedlings. With both of these species it has also been difficult to obtain adequate natural or artificial regeneration (Boyette 1980, Hill 1986).

The new national initiatives on ecosystem management, restorational ecology, biodiversity, and sustainable multiple use management will increase the demand for hardwood seedlings. This will be especially true with the heavy-seeded hardwoods like the oaks, which are important sources of wild life food as well as timber.

Some individual members of the genus Quercus grow well over a surprisingly narrow range of sites. One species may be found primarily on the most xeric upland Piedmont sites, another on the best mesic sites, and still another in

bottomlands. Thus, the genus is ubiquitous over a wide range conditions, but the individual species are not.

If the nursery requirement of seedlings from different sites paralleled their natural ones, production of oak seedlings of many species would be very difficult and expensive. Fortunately, the results of the preliminary nursery study described here indicate that oak seedlings of many species can be grown under nearly identical nursery conditions.

At the USDA Forest Service's, Institute of Tree Root Biology (ITRB), we have been modifying and testing various nursery practices in conjunction with research on root morphology and first-order lateral root (FOLR) development on various hardwoods and conifers (Kormanik and Muse 1986, Ruehle and Kormanik 1986, Kormanik et al. 1989, 1990). We have placed considerable emphasis on clarifying how plant metabolism and soil management practices affect root and stem development (Kormanik et al. 1992, Sung et al. 1989, 1993).

While our early efforts concentrated on sweetgum (Liquidambar styraciflua) and loblolly pine (Pinus taeda), we also studied oaks at the Whitehall Experimental Nursery. We found that we could grow most hardwoods and conifers with a single soil fertility baseline for Ca, P, K, Mg, Cu and Zn if we modified the nitrogen and irrigation practices to accommodate individual species (Kormanik, et al. 1992). In 1986, we began trying to adapt our research findings for hardwoods at the three forest tree nurseries operated by the Georgia Forestry Commission.

Typically, only 3 or 4 species of oak were available in any given year, and the numbers of each were usually limited. While the fertility baseline may differ somewhat among nursery locations, we found that amount and timing of nitrogen and water applications were the keys to production of high-quality oak seedlings. Nursery practices for a few species became nearly routine, but we wanted to be able to grow a broad range of oak species. We decided to try and test a series of species needed for restorational ecology efforts at the U.S. Forest Service's Savannah River Forest Station, Aiken, SC, from a wide range of environmental and edaphic conditions.

OBJECTIVE

The objectives of our study were: (1) to determine if a single nursery management protocol could be used for growing many species of oak in a single nursery, (2) to determine the numbers and positions of FOLR on the taproot, and (3) to compare morphological characteristics of species with diverse site requirements.

METHODS

In October-November acorns were collected by GFC personnel from randomly chosen individuals throughout the state. The acorns from a given species were composited. Altogether acorns were collected from 15 species of oak: Quercus alba (white), Q. falcata var. falcata (southern red), Q. falcata var. pagodifolia (cherrybark), Q. lyrata (overcup), Q. macrocarpa (bur), Q. michauxii (swamp chestnut), Q. nigra (water), Q. nuttallii (nuttall), Q. palustris (pin), Q. rubra (northern red), Q. shumardii (shumard), Q. stellata (post), Q. velutina (black), Q. virginiana (live), and Q. laurifolia

(Darlington). The acorns were kept in the cold storage facilities at the GFC's Morgan Nursery at Byron, GA, until they were hand-sown in December.

Based upon research at the three Commission nurseries and the Institute's experimental nursery, a single soil fertility level was chosen that has provided good seedling responses for all hardwoods tested. The soil fertility baseline had been developed by applying or withholding fertilizers over a 3 year period. All soil analyses were obtained from the A&L Laboratories, Memphis, Tennessee.

Soil levels of Ca, K, P, Mg, Cu, Zn, and B were adjusted to 500, 80, 50, 0.3-3, 3-8 and 0.4-1.2 ppm, respectively. Nitrogen was applied as NH_4NO_3 at rates equivalent to 1345 kg/ha (1200 lbs/acre). The first two applications were at a rate equivalent to 17 kg/ha (15 lbs/acre), the third at 56 kg/ha (50 lbs/acre). The next six applications were at 168 kg/ha (150 lbs/acre) and the final two at 112 kg/ha (100 lbs/acre). Nitrogen applications started in mid-May were continued at 10-day intervals until mid-September.

The acorns were hand-sown for a density of 54 to 57/m² (5 to 7 ft²) but the number of acorns available for each species varied widely. We had 10 to 20 times as many of the more commonly collected species, like northern red and white oak, as we had of less common species like Darlington oak. Accordingly, we sowed the beds continuously with a single species until all its acorns were gone, left a 2 to 3 m (6 to 10 ft) space and started sowing another species. The total area used directly in this study was approximately 5% of the entire field sown to many different hardwood species as well as to other oak seedlots.

While the root systems became established, seedlings were provided regular small daily applications of water. After the first growth flush was essentially completed and seedlings were into their second flush, water was applied when the moisture tensionmeters registered 30-50 centibar at 15-20 cm depth (6 to 8 inches). A total of 24 cm (9.5 inches) of irrigation water was applied during the growing season. Irrigation was essentially discontinued by mid-October. Rainfall of 91 cm (36 inches) was well distributed from April through December and only in October were there 3 weeks of below average rainfall.

The seedlings were undercut to 25-30 cm (10 to 12 inches) and lifted in mid-February. One hundred seedlings of each species were randomly selected for measurement of root collar diameter (RCD), height, and number of FOLR greater than 1 mm in diameter. Distribution of FOLR along the taproot and mycorrhizal development on fine feeder roots also were observed.

Our intent was to observe how each species developed under the nursery protocol. We planned no species comparisons, and we made no effort to quantify genetic variation by keeping track of mother trees. Seedlots were mixed as they are in normal hardwood nursery operations.

RESULTS AND DISCUSSION

This research, earlier trials, and subsequent yearly nursery tests in 1991 and 1992 at different GFC nurseries have demonstrated that it is feasible to use a single nursery fertility baseline nursery for hardwood seedling production once the background fertility level has been established at the

specific nursery. This approach works for a multitude of oak species, regardless of their environmental and edaphic requirements. This finding alone should significantly simplify and enhance oak seedling production.

Nitrogen and Irrigation Management

Oaks (and hardwoods in general) require higher soil fertility than do many pines in the Southern United States. After the soil fertility baseline is established at a specific nursery which may take 2 or 3 growing seasons, the primary management inputs are nitrogen and water. Rather large oak seedlings are desirable and growing them requires adequate nitrogen. Oaks form mesic and hydric sites require nitrogen applications at 10- to 12-day intervals to maintain continuous elongation, and the species from xeric sites also reach their maximum size with this consistent supply of nitrogen.

Usually throughout the summer 20 to 30% of the seedlings are in the flushing phase. It takes several weeks for a given flush to mature before physiological restraints permit another flush to occur. If during this resting stage nitrogen and water are limiting, subsequent flushes may be severely restricted and the elongation of the current flush is reduced. During resting periods between flushes roots grow and expand. We have counted five distinct "growth rings" on the taproots of northern red oak seedlings that have undergone six growth flushes during a 26- to 30-week nursery growing season.

Most nursery operators irrigate when they think it is necessary or on some predetermined schedule such as a given amount of water per week. Consequently, nurseries more often are over than under-irrigated. Over watering is expensive and it can leach away K and N. In addition, most young seedlings have a poor tolerance for wet feet. In general soils with a sandy texture need water in mid-summer more frequently than do soils with more clay and silt. By irrigating from tensionmeter readings leaching losses of essential elements can be reduced and the guess work can be removed from the process. For several years, we have been observing that southern red oak leaves seem to wilt on clear, hot and humid days soon after irrigation even though tensionmeters are registering less than 20 centibars. Other oaks do not begin to show stress until several days after the tensionmeters have reached 70 centibars. Thus, seedling appearance is not a good indicator of the need to irrigate.

General Observations

The observed ranges in FOLR numbers, heights, and RCDs are shown in Table 1. The means for this data set plus percentages of seedlings with less than the mean number of FOLR for the species are shown in Table 2. Table 3 includes comparable data for comparative purposes from 3 different years when fertility trials were being run at the ITRB experimental nursery.

Table 1. Ranges in numbers of first order lateral root (FOLR) and stem characteristics for 15 species of oak grown in the Georgia Forestry Commission Nursery - 1991.

Species	Range FOLR	X Height range (cm)	X RCD range (mm)
Southern Red	0-9	26-59	4.9-8.7
Live	1-12	28-76	3.8-9.7
Darlington	0-13	14-60	3.5-11.7
White	0-21	21-80	4.4-15.8
Water	0-18	21-63	2.9-11.8
Bur	0-29	27-114	5.8-19.5
Swamp Chestnut	0-19	33-80	6.3-21.2
Cherrybark	0-17	52-112	4.1-12.6
Post	0-21	31-89	6.5-14.3
Nuttall	0-20	42-138	5.5-19.9
Pin	1-21	31-120	5.6-20.5
Northern Red	1-23	24-143	5.0-14.6
Black	1-22	38-147	6.7-15.6
Shumard	1-27	65-165	5.7-14.1
Overcup	0-32	61-137	7.0-24.7

Table 2. Mean number of first-order lateral root (FOLR), heights and root collar diameters and percentages of seedlings with less than mean number of FOLR for 15 species of oak in 1991 nursery trial.

Species	X FOLR number	% Seedlings <X FOLR	X Height (cm)	X RCD (mm)
Southern Red	2	46	42	6.2
Live	3	57	44	5.6
Darlington	4	41	35	7.7
White	4	52	35	7.5
Water	5	53	39	6.4
Bur	5	53	54	9.9
Swamp Chestnut	6	60	48	10.5
Cherrybark	7	45	95	9.1
Post	7	53	73	10.7
Nuttall	8	38	94	13.0
Pin	9	45	66	11.8
Northern Red	9	56	71	9.0
Black	10	47	95	11.0
Shumard	11	52	128	11.3
Overcup	13	48	99	14.7

Table 3. Ranges and means for number of first order lateral root (FOLR) and stem characteristics for three species of oak grown in the Whitehall Experimental Nursery.

Year and Species	X FOLR	% Seedlings <X FOLR	Range	X HGT (cm)	Range (cm)	X RCD (mm)	Range (mm)
1986 Cherrybark ^a	5	54	0-17	83	20-152	6.63	2.4-14.2
1987 White ^b	7	53	0-34	34	6-111	7.50	1.5-15.5
1988 Northern red ^a	8	56	0-41	74	9-144	8.62	2.2-14.5

^a Average of 200 seedlings from each of 12 open-pollinated half-sib progeny.

^b Average of 200 seedlings from each of 3 open-pollinated half-sib progeny.

Generally species from more xeric sites had fewer and smaller ranges in FOLR numbers than species from mesic or hydric sites. This reduced number of FOLR was associated with overall smaller RCDs and less total height (Table 2). Earlier we reported (Ruehle and Kormanik 1986, Kormanik et al. 1989) that perhaps less than half of the oaks produced in our nurseries may be competitive in the field. How to identify these potentially competitive oak seedlings must be determined, after outplanting but we have found that, the greater the number of FOLR in the nursery, the more competitive the seedling.

Although we will have to await the results of outplanting trials, we suspect that individuals producing fewer FOLR than the average for the species will perform poorly in the field. We think this rule of thumb will hold together even when the mean FOLR is 2 as in southern red oak or 11 as in overcup oak. Thus, the percentage of seedlings with less than the average FOLR number (Table 2) may be extremely important. Even with different growing conditions in different years, the percentage of seedlings with fewer than the mean number of FOLR remained reasonably consistent (Tables 2 and 3).

Species Performance

Observations with these 15 species were obtained from a single nursery. However, six of them (northern red, white, swamp chestnut, cherrybark, southern red, and water) have been followed regularly for 5 to 6 years at the other nurseries. The Morgan nursery was simply the first where the fertility baseline was attempted on a large scale and it took about 3 growing seasons to bring the various nutrients into the balance we were striving to attain.

It appears that as long as bed density remains reasonably near the specific limits (54 to 57/m²) root relationships observed here remained relatively

similar. Only when seedlings are excessively stressed or grown at higher densities will the root morphology be significantly altered.

Our goal in these nursery trials is to learn how to grow oak seedlings to average heights of 0.75 to 1.40 m. Increasing irrigation and nitrogen applications, have not significantly increased heights of the xeric site oaks. However, under higher luxuriant conditions, the other oaks tended to get larger than we desire at the present time.

The only xeric species we have studied over several years has been southern red oak. In all cases, FOLR root development has been poor and mycorrhizal development has been virtually absent. In all cases, swamp chestnut and cherrybark oak have become heavily mycorrhizal and northern red and white oak have moderate but good mycorrhizal development.

The observed attributes of individual species are listed below:

Southern red oak.--Very few FOLR were produced. This absence of FOLR production has been noted in several other seedling trials. The greatest carbon allocation in seedling roots appeared to go into taproot development. Mycorrhizal development was very poor even on individuals that produced several FOLR. Individuals with the highest number of FOLR were the most competitive. Study of this species will be interesting and challenging.

Live oak.--Development of FOLR was either good or very poor. Intermediate development was lacking. The taproot may be a critical storage sink. Few if any mycorrhizae were observed on any seedling. A pronounced bulbous swelling was formed about 2.5 to 4.0 m below the root collar. Cutting it half revealed soft tissue with the consistency of a potato. Some of the smallest individuals with few, if any, FOLR had the largest swellings.

Darlington oak.--Over 80% of the seedlings had no FOLR in the 6 inches directly below the root collar. The few FOLR that were present were frequently 4 to 6 mm in diameter and could support the entire seedlings weight with a minimum of flexing. Mycorrhizae were essentially absent. The few we found were at the root collar and were not associated with the lateral roots. Many seedlings had multiple tops and the leaves still had not developed abscission layer in February.

White oak.--As in earlier trials, this species has displayed a wide range in rootsystem development. The very best 10 to 20% had a large diameter taproot with many FOLR. Another 20 to 30% seedlings had large diameter taproots with few FOLR. The stem characteristics of these two groups comparable, but seedlings with the greater number of FOLR had larger RCDs. The remaining seedlings had few FOLR and small diameter taproots. They were consistently non competitive in the nursery. Small isolated patches of mycorrhizae were present on the seedlings with many FOLR.

Water oak.--Individuals with few of FOLR were were least competitive in the beds. Seedlings with the large RCD were the tallest ones. Those with few FOLR had unacceptably small RCDs. Leaf morphology ranged widely. Few mycorrhizae were observed on any seedling.

Bur oak.--The relationship between seedling size and FOLR numbers was very strong. Only walnut seedlings have been observed with such large diameter taproots. The 10 to 20% with these large taproots and high FOLR numbers with FOLR diameters of ca 3 mm were really impressive seedlings. Perhaps 25% had large diameter taproots but few FOLR. The seedlings with small diameter taproots had few FOLR, small RCDs, and unacceptable form. Most seedlings had 5 flushes, but the poorest seedlings with ≤ 3 FOLR normally had 3 flushes. Small patches of mycorrhizae were found on only two seedlings.

Swamp Chestnut.--Assessment FOLR development was very difficult on this species. In addition to lateral roots with diameters larger than 1.0 mm there were countless others with diameters of ca 0.25 mm. Both the large and small lateral roots were so heavily mycorrhizal that the taproot was not readily visible without moving the dense mycorrhizal complex. Small seedlings with few ≥ 1 mm FOLR had considerably fewer mycorrhizal feeder roots. The individuals with massive root systems all had 5 to 6 growth flushes, while those with few roots had 3 to 4 flushes. Most of the seedlings with only 1 to 3 FOLR not only had inferior stem development but also had badly deformed taproots. Some taproots "corkscrewed" 1 to 3 times in complete 360 degree circles. This condition inhibited both stem and root development. Such taproot deformity has since been found on this species in all trials where it has been included.

Cherrybark.--On individual seedlings, FOLR diameters ranged widely. It was not unusual to have diameters from 1 to 7 mm on the same seedlings. Almost all the FOLR were on the first 20 cm of the taproot. Even though the nursery soil was not excessively moist, many seedlings had development water roots that appeared to emerge from proliferated lenticels. All of these water roots were unbranched and some were up to 24 cm long. Some suberized FOLR with diameters of 1.5 mm to 2.5 mm developed long water root extensions. The largest seedlings had the best developed FOLR and had up to 6 or 7 growth flushes. Mycorrhizae developed along the entire lengths of FOLR, but development was not nearly as dense as with swamp chestnut oak.

Post.--A high percentage of the trees had forked or multiple tops leading us to wonder whether the acorns were from a single mother tree. Multiple stems developed rather consistently at the beginning of the fourth flush. Most seedlings had 4 or 5 flushes; the very poorest had only 3. As with Darlington oak, FOLR were absent within 20 cm of the root collar. In most oak species FOLR development begins just below the root collar. Mycorrhizae were observed on only 8 seedlings, and their development was spotty and sparse.

Nuttall.--FOLR had rather large diameters of up to 9 mm and were uniformly distributed along the entire excavated taproot. Not all seedlings had water roots, but those that did had unbranched ones, that often were 15 to 20 cm long. The largest diameter and tallest seedlings had the very large diameter FOLR. The relationship between FOLR development and stem diameter and height growth was close. Most individuals had 5 to 7 flushes but even the smallest ones had at least 4 flushes. Mycorrhizal development was uniform and dense along the entire lengths of mature FOLR. Even taproots had uniform and dense mycorrhizal short roots.

Pin.--Individuals either had a robust root system with abundant FOLR and a stocky stem or few FOLR and a short stem with a small diameter. Thus, the junk was easy to pick out. Good seedlings would be extremely difficult to ship,

however, because of the large diameter FOLR (up to 12 mm) and excessively long laterals. FOLR up to 90 cm long were excavated. Even if they were held near their distal end, they supported the entire seedling's weight. Mycorrhizal development was present on only a few seedlings, and it was sparse and light.

Northern red.--FOLR were concentrated in but not limited to the top 20 cm of taproot. Competitive seedlings all had large numbers of FOLR and had between 5 and 6 flushes. Individuals with few FOLR had 2 to 3 flushes. Mycorrhizal development was scattered but moderate where it occurred. Most FOLR diameters were in a narrow range from 1 to 4 mm. Seedlings of this species may have had the most striking differences based on FOLR numbers.

Black.--Root systems had large diameter taproots and many well-developed FOLR. The FOLR diameters varied from 1 to 7 mm and this range frequently occurred on an individual seedling. Mycorrhizal development was infrequent and sparse. The better seedlings seemed ideal for planting. The best 50% of the seedlings should be competitive.

Shumard.-- FOLR diameters ranged from about 1.5 to 3.0 mm. On individual seedlings, FOLR diameters were the most uniform of any observed on any species. All seedlings, regardless of size, had 4 to 5 growth flushes. Mycorrhizae were abundant and dense but short roots were very thin and delicate. The mycorrhizal feeder roots dropped off very quickly after the roots were excavated. Abundance of mycorrhizae therefore was assessed at lifting and not when FOLR were counted and growth data were collected.

Overcup.--Of these 15 species of oak, overcup had undoubtedly the best and most uniformly developed FOLR. Taproots often were 1 1/2 to 2 times larger in diameter than root collars. The FOLR were 1 to 3 mm diameter and they remained flexible even though they were 1 to 2 m long. Water roots up to 5 cm long and 3 mm in diameter were present. They were "crisp" and popped like a celery stick when broken. Mycorrhizae were abundant and dense throughout the length of the FOLR and occurred in clusters along the taproot. The mycorrhizae clusters were so abundant that it was difficult to remove the attached soil to examine the FOLR morphology. This was the only species in which we would not cull individuals with fewer than the mean FOLR number. The seedlings all consistently had just 5 flushes, and less than 10% would have been considered culls by the authors.

CONCLUSIONS

Our results demonstrate that a multitude of oak species can be grown in a single nursery with one management protocol. It is equally evident that more nitrogen and close monitoring of water use will be required to maintain continued stem and root development throughout the growing season.

This and subsequent work also show species-specific growth patterns among oaks. Seedlings of species, normally found on xeric sites will not be as large as those species found on mesic and hydric sites. Species typically found on moist bottomlands or seasonally hydric sites have better developed lateral root systems. These species also have the best mycorrhizal development. The reason for species difference in mycorrhizal development in the nursery was not readily apparent but there was a gradient in abundance with xeric < mesic <

hydric sites. The gradient has been observed in other trials but its biological significance has not been clarified.

On species that occur in moist wetlands water roots developed in the nursery beds in late fall even though the beds were not saturated. This phenomenon deserves additional study.

Competitive individuals of all species - those in dominant and codominant canopy positions - had more than the mean number of FOLR for the species in question. Based on competitiveness and root development in the nursery, it appears that about half of oak seedlings may not be competitive after outplanting.

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MICROPROPAGATION OF MATURE RED MAPLE (*ACER RUBRUM* L.)

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Abstract.--Micropropagation of mature red maple was achieved from axillary buds of both dormant and greenwood branch cuttings. Axillary bud break and multiple shoot formation occurred on MS medium containing 0.01 mg/L thidiazuron (TDZ), as benzyladenine (BA) proved to be inhibitory to shoot proliferation and elongation. Low light intensity and culture system were critical to successful micropropagation, as shoot cultures maintained at 150 ft-c on microporous polypropylene membrane "rafts" produced a 10-fold increase in the number of shoots suitable for rooting when compared to cultures maintained on agar-solidified medium at 450 ft-c. For most genotypes tested, roots were visible in as little as six days on shoots given an auxin pretreatment *in vitro*. Rooting of shoots and establishment of plantlets in soil occurred at an overall frequency of 77%.

Keywords: *Acer rubrum* L., micropropagation, tissue culture, plantlets.

INTRODUCTION

Red maple (*Acer rubrum* L.) is the most widely distributed hardwood species in the Eastern United States, being found in virtually all states east of the Mississippi River as well as Southern Ontario. In coastal regions, its range spans from east Texas to Newfoundland. Red maple is one of the most frequently planted landscape trees in municipalities in the United States, and is especially appreciated for its fall color. Based on fall color, several commercial cultivars (e.g., 'Red Sunset', 'October Glory' and 'Autumn Flame') are well-known. In the Southern United States, red maple is abundant in river bottom hardwood forests. Red maple can become an important component of pulpwood furnishes for paper mills in this region, especially during dry summers.

Of all hardwood species present in the Southern United States, red maple is perhaps the most outstanding fiber source for the manufacture of bleached grades of paper. In bleached grades, used primarily for writing and printing papers, the emphasis is on the optical properties (such as opacity) and printing properties (such as smoothness). In general, the paper properties of smoothness and opacity increase as the coarseness (fiber mass in milligrams per 100 meter length) of the papermaking fibers decrease. Fiber coarseness, in turn, is strongly influenced by fiber length (Clark, 1978). At a fiber length of 0.82 mm (Isenberg, 1951), red maple has the shortest fiber (and, consequently, the lowest coarseness) of all the major hardwood species used for pulpwood in the Southern United States. Given the recent emphasis on paper quality, and, that virtually all new paper manufacturing capacity in the South is in bleached grades, red maple is a species eminently suited for the goals and objectives of the fine paper manufacturers.

Red maple is a good candidate for vegetative propagation for several reasons. Red maple cannot be easily grown from seed using conventional nursery practices developed for many other hardwood species (R. Heeren, personal communication). Part of the problem stems from the fact that it is one of the few species that seeds in the late spring. Because the seed is fleshy and does not store well, it must be collected, processed, sown, and seedlings grown, all in one season.

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Vegetative propagation would be one way to avoid this logistical problem. In addition, experience with red maple seedlings grown in an open nursery bed has shown that they tend to be tall and thin, with poor root collar diameters, and poor survival after establishment. Vegetative propagation may be one way to produce higher quality planting stock by setting cuttings of larger caliper, and, thereby increasing root collar diameter over seedlings.

In addition to logistic and planting stock quality considerations, the lack of genetically improved material suggests that vegetative propagation may represent the fastest and most cost-effective way to exploit the best individuals currently available. Finally, red maple is a species amenable to vegetative propagation, as propagation by rooted cuttings is used commercially to produce large numbers of the cultivars noted above (MacDonald, 1989).

Red maple micropropagation was undertaken as a means of rejuvenating mature trees in the field and producing stock for test plantings. Red maple micropropagation has been previously reported for a specific commercial cultivar 'Red Sunset' (Welsh, 1986; McClelland and Smith, 1990), and for hybrids between red and silver maple (*Acer x freemanii*; see Kerns and Meyer, 1986). However, published procedures employed only greenwood cuttings collected from grafted individuals. In particular, no information was available on how field-grown material would respond in culture, or on how the timing of collection would influence the ability to establish cultures and induce multiple shoot proliferation. In addition, the number and quality of microshoots suitable for rooting produced by cultures grown on published media formulations was lower than desired. For these reasons, an effort was undertaken to develop a more versatile and efficient micropropagation system for red maple which could be extended to a large number of genotypes from throughout its range.

METHODS

Plant Material and Culture Initiation

Explants (dormant, axillary buds or greenwood scions) were collected from locations in Virginia, South Carolina, and New Jersey. Dormant buds were collected from sexually mature trees ranging in age from 20 to 40 years. Greenwood scions (for establishment of cultures from nodal segments) were collected from young saplings or seedlings. Dormant buds were collected from the period of July-February, and nodal segments from greenwood cuttings were used to establish cultures from April-May. Dormant buds were removed from branches and rinsed under tap water to remove surface dirt. (Note: axillary buds from mature trees are often three-lobed; only the center lobe was sterilized, as the two outer lobes are flower buds.) With the outer bud scale still attached, dormant buds were given a treatment 10% Wavicide (a hospital disinfectant containing 2% glutaraldehyde; Wave Energy Systems, Cedar Grove, NJ) for 10 min. Under aseptic conditions, buds were then treated with 20% household bleach (1.1% sodium hypochlorite) for 20 min, employing three drops of Tween 20 per 100 mL solution as a wetting agent. Following three rinses with sterile water, the outer bud scales were removed under aseptic conditions, and the buds were treated with 2.5 % bleach for 10 min, rinsed three times with water and then treated 0.1% HgCl₂ for 5 min. After another three rinses with sterile water, the remaining bud scales were removed and the shoot tips were treated with 1% household bleach for 5 min, rinsed three times, and were plated onto culture media. For greenwood explants, branch segments (1 cm in length) containing a single node (i.e., two axillary buds) were treated with 20% bleach for 20 min, rinsed three times with sterile water, soaked for 1 h in sterile water, and were plated onto culture media.

All culture media was solidified with 0.7% bacto agar, and cultures were maintained at 26°C under 1,200 $\mu\text{W}/\text{cm}^2$ illumination (cool white fluorescent) on a 16 h photoperiod. For both

dormant buds and greenwood cuttings, the media contacting the explants darkened very soon after initiation. To minimize any deleterious effects of this dark exudate, the explants were transferred to a different part of the plate that had no visible darkening. Transfer was done three days after initiation, and again after six or seven days. After this time, cultures were transferred to fresh media every four-six weeks.

Shoot Elongation and Root Formation (Best Way)

Shoot cultures were routinely initiated and maintained on MS medium containing 0.01 mg/L thidiazuron as the only growth regulator. For the production of long shoots suitable for rooting, cultures were transferred from agar-solidified medium to microporous polypropylene membrane "rafts" (Sigma Chemical Co., M-1049) placed over liquid medium of the same composition. After four to six weeks, microshoots (1-2 cm) were harvested for rooting. Cultures from which shoots were harvested were returned to agar-solidified media for continued growth.

Shoots were set for rooting in one-third strength WPM media (macro- and microelements) containing 0.1 mg/L indolebutyric acid (IBA) and 1% sucrose. Roots typically were visible in as little as six days. Rooted shoots were set in a soil-less mix containing equal parts peat, perlite and vermiculite, and were kept in a plexiglass "fog box" at 100% relative humidity. Plantlets were kept under high humidity at $2,700 \mu\text{W}/\text{cm}^2$ under a 16 h photoperiod until shoot growth resumed, usually within four weeks or less. After this time, plantlets could be maintained under the ambient conditions of a greenhouse. Planting stock for field testing was produced by transplanting plantlets (4-5 cm) to 10 in³ Leach tubes in the spring, growing the plantlets all summer in a greenhouse, and hardening them to outdoor conditions in September. Plantlets (30-50 cm) with well-developed root systems were overwintered at 4°C prior to establishment in the field.

RESULTS AND DISCUSSION

Establishment of shoot cultures in red maple was influenced by the season in which explants were collected (see Table 1). Shoot proliferation from nodal segments taken from greenwood cuttings collected in the spring proceeded rapidly (three weeks), and was induced in over 80% of the explants cultured on MS media containing 0.01 mg/L TDZ. In addition, every genotype cultured from nodal segments collected in April and May could be established in culture. Previously published reports on micropropagation of 'Red Sunset' or red x silver maple hybrids have capitalized on this observation, and report exclusively on the use of nodal explants taken from greenwood cuttings collected in spring or early summer from grafted individuals. Our findings show that grafting is an unnecessary step, as greenwood cuttings can be collected from the field and established in culture with little difficulty. The rapid rate of shoot proliferation from explants collected in the spring means that plantlets could be established in soil from a mature tree in as little as five months.

Table 1. Effect of collection date on the establishment of shoot cultures in red maple.

Month	Genotype Origin	Genotypes, No.		Time To Shoot Proliferation, Wks.
		Collected	Established <u>In Vitro</u>	
February	VA	4	2	12
May	VA, NJ	4	4	3
July	SC	5	2	8
September	VA, NJ	5	3	8
December	VA	4	2	12

On the other hand, explants from greenwood cuttings that had set bud in July were more difficult to establish in culture and took up to eight weeks. Axillary buds had to be explanted from these cuttings, as culture establishment from the nodal segments was unsuccessful. Although axenic cultures could be established from excised buds from greenwood cuttings, subsequent shoot elongation and proliferation was not as rapid and vigorous as from actively growing greenwood cuttings. Indeed, even though four of the five genotypes were established in axenic culture, after six months only two of the genotypes went on to produce rapidly proliferating shoot cultures. Seasonal variation in the rate and extent of shoot proliferation from bud explants of woody perennials has been previously documented, and is thought to be related to the depth of internal dormancy (Fukui et al., 1990).

Multiple shoot proliferation from axillary dormant buds was also slow. At the various times that cultures were initiated from this explant (September, December and February) 8-12 weeks were required before multiple shoot formation was observed. Unlike greenwood cuttings, no seasonal effect on shoot proliferation was observed in cultures established throughout the dormant season. However, not every genotype examined could be established in culture, mainly due to losses associated with contamination, or from damage caused by the sterilization treatment used. In many hardwood species (e.g., oak, aspen and sweetgum) there exists a ramified layer of cortex tissue subtending the apical meristem, as well as numerous layers of leaves surrounding the meristem. Both of these features, which can protect the apical meristem from damage due to the sterilizing agents, are missing in dormant red maple buds. Without the protection afforded by numerous leaves and cortical tissue, establishing axenic cultures from dormant buds can be difficult.

Thidiazuron (TDZ) has been reported to be critical to shoot proliferation in tissue cultures of *Acer x freemanii* (Kerns and Meyer, 1987), and this was also observed in red maple (see Table 2). When used alone or with low concentrations of BA, a concentration of TDZ at 0.01 mg/L was near optimal, as 0.1 mg/L TDZ caused excessive basal callus formation at the expense of shoot proliferation. As the BA concentration was increased to 2 mg/L in the presence of 0.01 mg/L TDZ, shoot proliferation was again inhibited and replaced by basal callus formation. At 0.001 mg/L TDZ, no shoot proliferation was observed (result not shown). New shoot formation from single-node shoots placed vertically on TDZ-containing medium followed two pathways. First, two new shoots elongated rapidly from the two axillary buds. Second, several adventitious shoots differentiated more slowly from the callus that formed at the base of the original shoot. As adventitious shoots appeared, their axillary meristems would, in turn, elongate. The result was that at the end of a six week period, it was not unusual to have up to eight new shoots (i.e., two axillary; two adventitious, with their four axillary shoot beginning to elongate) from a single shoot. Therefore, micropropagation of red maple using this procedure yields a mixture of axillary and adventitious shoot types. Although the possibility exists of off-types being produced via an adventitious pathway, no phenotypic variants have been observed among the hundreds of plantlets produced to date.

Table 2. Effect of benzyladenine and thidiazuron on shoot proliferation in red maple (three genotypes; four week evaluation).

		Number of New Shoots/Shoot			
		0-----	0.1-----BA, mg/L-----	1.0-----	2.0
	0	30% Rooting	0.2 ± 0.1	0.13 ± 0.05	Callus
TDZ,	0.01	4.1 ± 1.5	3.5 ± 1.2	3.5 ± 1.3	Callus
mg/L	0.1	Callus	Callus	Callus	Callus

Used alone, the widely-utilized cytokinin BA did not support shoot proliferation. Instead, at increasing concentrations, BA stimulated the formation of large amounts of basal callus from the cut end of excised shoots. At lower concentrations (0.01-0.025 mg/L), the ineffectiveness of BA was underscored by the observation that shoots rooted quantitatively (results not shown). Inclusion of low concentrations of cytokinin to in vitro rooting treatments can produce higher quality plantlets by promoting shoot health during rooting; this should not be overlooked as a means of increasing plantlet survival, especially in difficult-to-root species (K. Louis, personal communication, 1993).

Red maple shoot cultures maintained under light regimes typically used for woody plant tissue cultures (i.e., 1,200 $\mu\text{W}/\text{cm}^2$ cool white fluorescent, 16 h photoperiod) had a decidedly red coloration of both stem and foliage. Cultures exhibiting red coloration did not proliferate well, but more importantly, did not produce the long (greater than 1 cm) high quality shoots needed for rooting. A reduction in light intensity (effected by the use of three layers of cheesecloth) by two thirds (i.e., down to 400 $\mu\text{W}/\text{cm}^2$) greatly reduced the red coloration of the cultures and increased the number of shoots suitable for rooting in some genotypes (see Table 3). Although the increase in shoot elongation was not observed in all genotypes, the improvement in the overall appearance of the cultures was such that shoot cultures of all genotypes were routinely maintained at the reduced light intensity.

Table 3. Effect of light intensity on the shoot elongation for two red maple genotypes (4 week evaluation).

Genotype	Number of Long Shoots/Culture*	
	1,200-----Light Intensity, $\mu\text{W}/\text{cm}^2$ -----400	
'Jordan Pocosin'	13.7 \pm 4.0 a	8.8 \pm 3.0 a
'Jillcott'	4.2 \pm 0.8 a	1.0 \pm 0.4 b

* Within a row, means followed by common letters are not significantly different as determined by t-test ($p = 0.05$).

Enhanced growth rates of shoot cultures of woody species can often be achieved by the use of liquid culture. However, liquid culture often leads to shoot vitrification and reduced survival upon transfer to soil. A recent development in culture systems that offer the growth advantages of liquid culture, while minimizing vitrification, are microporous, polypropylene membrane "rafts" (Sigma Chemical Company, 1990). In this system, shoot cultures are placed on a porous, polypropylene support over a liquid media that allows for the diffusion of substances that might be inhibitory to growth, as well as the effusion of nutrients such that nothing becomes limiting to growth.

Table 4 shows the results obtained using the membrane raft system. Again, improvement in growth rate and shoot elongation with the membrane raft culture system was genotype dependent. In genotypes in which the membrane system improved growth rate and shoot elongation, it was not possible to maintain the cultures on the raft system for extended subculture intervals as vitrification would develop. However, the extent of vitrification could be controlled by alternating between the raft system for one cycle (4 weeks), followed by a four week cycle on agar before once again undergoing transfer to the rafts. Coupling the reduced light intensity with the membrane rafts could, with some genotypes, greatly increase the production of shoots suitable for rooting.

Table 4. Effect of culture system on shoot elongation of red maple (4 weeks).

Genotype	Shoot Elongation, No. Shoots > 1 cm		Growth Rate, gm	
	Agar	Membrane Raft	Agar	Membrane Raft
'Jillcott'	0	3.6 ± 1.1	0.31 ± 0.04	1.45 ± 0.48
'Jordan Pocosin'	8.8 ± 3.0	9.8 ± 2.6	ND	ND

Rooting and transfer to soil of red maple microshoots was remarkably easy. As noted, several media (WPM or MS) containing either no growth regulators, low concentrations (0.01-0.25 mg/L) of BA, or IBA (0.1 mg/L) promoted rooting, usually within as little as six days. All genotypes rooted easily, and no genotypic effects on root formation were observed. The ease of rooting implies that physiological rejuvenation has occurred (Howard *et al.*, 1989). The overall rooting and survival in soil of twelve genotypes in which planting stock has been produced averaged 77%. After one season's growth in a greenhouse, and overwintering, several small demonstration plantings have been established near Franklin, VA.

CONCLUSION

Micropropagation of mature red maple is one way in which selected trees can be rapidly propagated for evaluation of growth potential. The procedure described avoids the time-consuming step of grafting, and identifies spring and early summer as the optimum times for culture establishment. However, the procedure can also be extended to material collected throughout the year, although culture establishment after bud set in the summer should be avoided. The procedure developed was successfully applied to a wide variety of genotypes, and the remarkable ease of root formation suggested that some type of rejuvenation had occurred. Procedures for red maple micropropagation departed from those used for many Southern hardwoods in that BA, the most widely used cytokinin, was inhibitory to shoot proliferation, while TDZ was critical to success. Reduction in light intensity, although not crucial to successful micropropagation, dramatically improved the quality of shoots set for rooting. In hardwood micropropagation, when excessive shoot pigmentation or extensive basal callus formation is observed, consideration should be given to an investigation of alternative growth regulators and light regimes.

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ABSTRACT

Hardwood genetics research at the Institute of Paper Science and Technology (IPST) seeks to produce useful variants of elite Populus deltoides via genetic transformation. Among other pre-requisites, transformation requires large numbers of suitable explants and reliable regeneration of plants. Model clones, easily manipulated in culture, are ideal for such research. Elite clones of commercial value, however, often prove recalcitrant. Trials reported here sought to establish shoot cultures of an elite clone, St75. Trial 1 tested varying zeatin (Z) levels. Differences among Z levels were small, but more shoots tended to form at 1 mg/L Z. Few shoots elongated to usable size. Higher Z levels tended to forestall explant deterioration. Trial 2 reexamined promising Z levels, and attempted to reduce deterioration and promote elongation by biweekly or accelerated subculture (ASC). Shoot production was enhanced by ASC on 1 mg/L Z. Explants remained healthy and productive through 161 days. Trial 3 retested this treatment and evaluated gibberellic acid (GA) as a stimulus for elongation. ASC, once again, extended explant life. Explants given GA (1 mg/L) at 115 days produced more usable shoots than in most previous trials.

Keywords: organogenesis, cottonwood, tissue culture, zeatin, gibberellic acid

INTRODUCTION

Hardwood genetics research at IPST centers on producing useful variants of elite eastern cottonwood (Populus deltoides) via genetic transformation. Traits of interest include herbicide tolerance and enhanced auxin synthesis. Herbicide tolerance promises lower plantation establishment costs and increased growth. Enhanced auxin synthesis may influence fiber numbers and/or dimensions (Klee et al. 1987). Increased fiber numbers could raise yields; altered dimensions, e. g., longer fibers with thinner walls, could raise paper quality. Also, ability to alter auxin status at will would permit investigation of mechanisms governing fiber formation.

Developing useful variants requires ready access to large numbers of clean explants, reliable methods for establishing, maintaining, and multiplying cultures, efficient means for effecting transformation and selecting transformed materials, reproducible protocols for regenerating plants, and techniques for confirming genetic change, stability, and utility. Such requirements are readily met by model clones, i. e., those easily manipulated in culture. Models are useful for research, but methods eventually must work with elite clones of commercial value.

Recently, IPST research has focused on methods for establishing cultures from elite clones. Internodes, a readily available and desirable tissue source, are used as explants. Experimental approaches generally have followed

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those of Coleman and Ernst (1989, 1990a, b). Early results showed that internodes from elite clones formed shoots nearly as frequently as those from model clones (Dinus et al. 1992a). However, only small numbers of shoots forming on internodes of elite clones elongated to usable size. As a result, they could not be perpetuated or multiplied in culture.

This report presents findings from three experiments designed to overcome recalcitrance of a valuable elite clone. Each trial was designed to refine or add to treatments giving beneficial results in preceding tests. A model clone, noted for predictable and productive behavior, was used as a "positive" control. Various combinations of growth regulator types, concentrations, and application times as well as subculture frequency were tested in efforts to improve explant health and shoot production.

MATERIALS AND METHODS

Plant Material

Two clones were used throughout the three experiments. Model clone K417 (supplied by Dr. C. S. Prakash of Tuskegee University), was chosen because of ease of manipulation in culture (Prakash and Thielges 1989, Coleman and Ernst 1990a), southerly origin (Fulton Co., KY), and potential utility on sites of interest - bottomlands along the lower Mississippi and Columbia Rivers. Elite clone St75 from Issaquena Co., MS (Mohn et al. 1970) was supplied by Dr. B. J. Stanton (James River Corporation, Camas, WA), and was chosen for site adaptability, superior productivity, and high alpha-cellulose content (Olson et al. 1985). Both clones were grown in a greenhouse on a temperature and photoperiodic regime designed to promote year-round growth and ensure continued explant availability. Water was provided as needed, fertilizer was added weekly, and various pesticides were applied alternately across weeks to prevent and/or remedy problems. Ramets were hedged periodically to limit size, maximize explant numbers, and facilitate pesticide application. The same three ramets of each clone were used as explant sources in all trials. Detailed growing conditions are given in Dinus et al. (1992a).

Explants

Stems were cut just below the eleventh node. Leaves were removed and stems rinsed with tap water. Earlier research (Douglas 1984, Coleman and Ernst 1989, Dinus 1992a) showed that response varied with internode position or age. To increase response and predictability, the present work used only internodes six through nine. After cutting into individual stem segments, all materials were surface-sterilized according to the procedures of Coleman and Ernst (1989). Segments were then aseptically dissected into 5 mm internodal explants. Nodes were discarded. Explants were placed in liquid WNA medium (Coleman and Ernst 1989), supplemented with antibiotics (500 mg/L carbenicillin, 50 mg/L tetracycline, and 15 mg/L rifampicin), and shaken in the dark for 24 hours to minimize systemic bacterial contamination (Coleman, personal communication). After rinsing three times in sterile deionized water, explants were placed horizontally on culture media.

Media and Experimental Design

Explants were cultured on WNA medium as modified by Coleman and Ernst (1989). WNA was supplemented with 0.5 mg/L 2,4-D to induce callus formation (CIM) (Coleman and Ernst, 1990b), and with Z to promote shoot formation and

elongation (Coleman and Ernst, 1990b; Dinus et al. 1992a). Growth regulators used in each experiment are described below. WNA was used at pH 5.8, solidified with 0.25% Phytigel (Sigma Chemical Co., St. Louis, MO), and autoclaved at 121°C and 1.4kg/cm² for 20 minutes. For the first 28 days of culture, carbenicillin (500 mg/L) was included in all media, regardless of growth regulator content, to control systemic bacterial contamination. Carbenicillin, Z, and GA were filter sterilized and added after autoclaving. Media (20 ml) were dispensed into 100 x 20 mm sterile polystyrene Petri plates.

To the extent possible, individual replications were filled with explants from one stem of a particular ramet from each clone. When one stem did not yield sufficient explants for a replication, explants from the next harvested stem were used. All work on a replication was completed on a given day by the same operators. This proved logistically efficient and potentially reduced experimental error by associating any variability caused by ramet condition or explant processing with replications. Each combination of replication, clone, and treatment (one petri plate) with seven explants constituted an experimental unit, and observations were based thereon.

Culture was done in darkness at 22°C for 10 days, and then under a 16 hour/day photoperiod at 22°C with illumination from 40W preheat- rapid start agro-lite lamps (15 μ moles M⁻² s⁻¹ of photosynthetically active light). Cultures were moved to fresh media every 28 days unless indicated otherwise. Observations of contamination, callus production, shoot formation and/or elongation, and explant health were recorded every seven days until deterioration or death dictated termination. Usable shoots (5 mm or longer) were counted, and collected for other research every two weeks beginning at day 63. Harvested shoots were rooted as per Dinus et al. 1992a.

Trial 1 sought to examine effects of elevated Z levels, and to confirm findings from previous work. St75 explants were arrayed in a randomized block design with six treatments and eight replications. Treatments consisted of 4 days on CIM (CIM4) followed by transfer to WNA supplemented with 0.00, 0.25, 0.50, 1.00, 2.00, and 4.00 mg/L Z. After exposure to CIM4, K417 material was placed only on the 0.5 mg/L Z level, a treatment found to induce shoots in earlier work (Coleman and Ernst 1990b; Dinus et al. 1992a). Comparisons between clones were based on eight replications of this treatment.

Trial 2 reexamined promising Z levels, and attempted to improve explant health and shoot elongation via modified subculture regimes. For St75, the experiment initially was organized as a randomized block design with 4 treatments and 12 replications. Treatments included WNA without Z, and CIM4 followed by transfer to WNA with 0.50, 1.00, or 2.00 mg/L Z. To offset explant deterioration and stimulate shoot elongation, the standard subculture regime was altered at days 49 and 56. The 12 replications for each Z level were distributed equally and randomly among subculture treatments: accelerated subculture (i.e., biweekly rather than monthly transfer to fresh medium) beginning on day 49 (ASC49); accelerated subculture beginning on day 56 (ASC56); standard or monthly subculture to medium without growth regulators (NOGR); and the standard subculture regime (CONTROL). Thus, each combination of Z level and subculture regime (16 treatments) was replicated three times. For analyses from day 70 through day 154, this arrangement was considered a completely random design. K417 was employed to track agreement with earlier trials. Eight replications were pretreated with CIM4, placed only on 0.5 mg/L Z, and given the standard subculture regime.

The third trial was part of a larger test designed to contrast effects of Z with those of another growth regulator, thidiazuron. Only 1 mg/L Z plus ASC56 and subsequent manipulations are described here. Six replications of St75 and three of K417 explants were placed on CIM4 and transferred as usual to WNA plus 1 mg/L Z. ASC56 was implemented at day 56 and continued thereafter. Additional measures to stimulate elongation were taken on day 115. At that time, replications for each clone and treatment were distributed randomly between two supplemental treatments. Three replications of St75 continued on Z and ASC56, while three were transferred to WNA supplemented with 1 mg/L GA (Welander et al. 1989) and subcultured biweekly. This arrangement was considered a completely random design with two treatments (GA and no GA) and three replications. As a check, two K417 replications were given GA; the third remained on the original treatment. Data and usable shoots were collected through day 203.

Data Collection and Analysis

Observations for each experimental unit were summarized as percentages of explants forming callus, forming shoots (FS), deteriorating, and elongating shoots (ES) on days 63, 91, 112, and later where noted. Observations of some treatments were extended to allow greater opportunity for St75 shoot elongation and to maximize numbers of harvested shoots. Data were subjected to analyses of variance (Steel and Torrie 1960) for the designs described above, using the general linear models procedure of SAS Institute (1985). When main effects were significant, means were compared using Duncan's New Multiple Range Test. All tests of significance were made at $P = 0.05$.

RESULTS AND DISCUSSION

In the first trial, all K417 explants formed callus and shoots; ES averaged 80% or more over the course of the experiment. Such findings typify those from earlier trials (Dinus et al. 1992a, b). Time course analyses of development (Figure 1, K417) showed that K417 callus formation began between 7 and 14 days after start of culture. Parallel increases in FS and ES occurred roughly 14 and 35 days thereafter, respectively. Explants remained healthy, with usable shoots harvested at 14-day intervals through or beyond day 112.

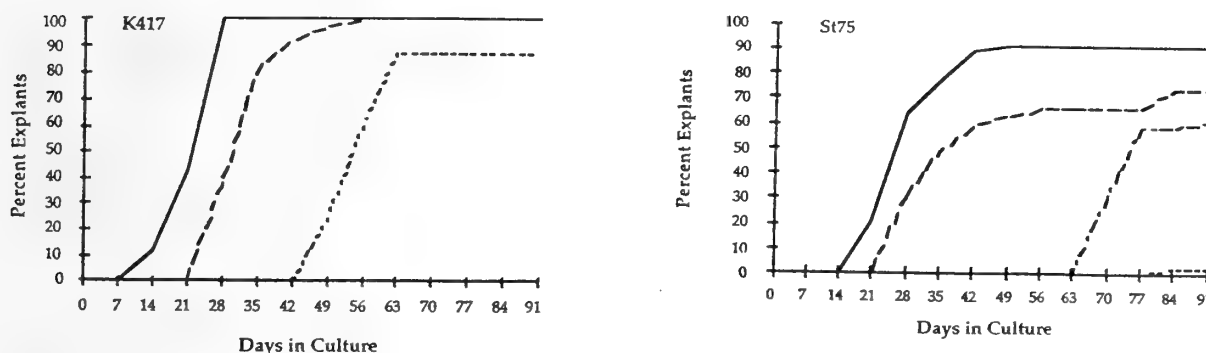


Figure 1: Time course of development for K417 and St75 on 0.5 mg/L zeatin over 91 days in culture. Callus formation——, Shoot formation— — —, Shoot elongation - - - - , Deterioration - . - .

Development for St75 followed a similar pattern, with some critical exceptions (Figure 1, St75). Callus and shoot formation occurred several or more days later than for K417, and neither attained levels as high as those for K417. Both callus and shoot formation frequencies varied somewhat with Z level, though differences were not significant through day 77 (Figure 2). Nonetheless, explants not receiving Z were least responsive, and those given 1 mg/L Z tended to form shoots more often. Later in the experiment, FS for explants receiving Z proved significantly greater than that for explants not given Z. Also noteworthy were the observations that St75 shoot elongation lagged behind that for K417 by roughly 35 days (Figure 1), and that it never exceeded 7 percent regardless of Z level (Figure 2). In addition, St75 explants began deteriorating on or shortly after day 63; browning, callusing-over, and death became increasingly frequent with time (Figure 1, St75).

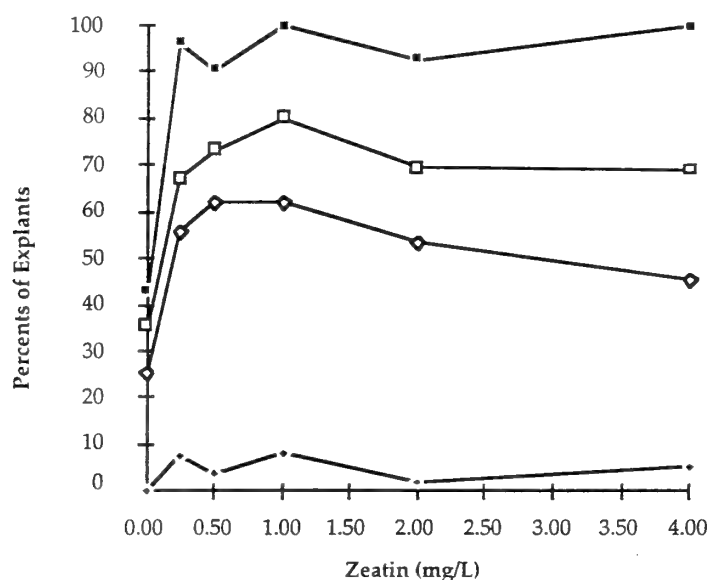


Figure 2: Responses of St75 explants to varying zeatin levels through 77 days in culture. Differences among zeatin levels were not significant. Callus formation \blacksquare , Shoot formation \square , Shoot elongation \diamond , Deterioration \bullet .

The 35 day lag in St75 shoot elongation and rapid decline of St75 explants suggested the need for some additional measure(s) to maintain explant health and/or stimulate elongation. The fact that 1 mg/L Z tended to raise FS, while higher Z levels tended to lessen deterioration (Figure 2) further suggested that moderate but more uniform Z levels might improve health and/or promote elongation. Though FS was significantly lower for St75 than for K417 (Figure 1), 60 percent or more of St75 explants had shoots available for elongation by day 49. Such findings infer that shoot elongation requirements differ from those for formation. Accordingly, subsequent experiments sought to reevaluate the more promising Z levels, and to intervene in the window of opportunity between peak shoot formation (day 49) and onset of deterioration (day 63).

Results from the second trial largely confirmed those from the first. K417 FS and ES matched earlier levels, and exceeded those of St75 by the usual margins. Performance of St75 through day 63 was also in agreement, but later results differed markedly. On average, St75 explants produced more usable shoots, and shoot elongation occurred over a longer period of time than in earlier trials. Proportionately larger numbers of explants remained healthy and productive through day 112, an average over all treatments of 13.2 percent relative to a maximum value of 7.0 percent for Trial 1.

One notable similarity occurred in that the 1 mg/L Z treatment, when averaged across all subculture treatments, gave significantly greater shoot elongation than other treatments. By day 112, roughly 33 percent of explants given 1 mg/L produced usable shoots, a frequency almost three times greater than the next highest (12 percent). Significant differences also occurred among subculture regimes, when averaged over all Z levels. Though similar to most regimes, response to ASC56 significantly exceeded that to NOGR (19.8 versus 1.7 percent). All regimes except NOGR produced more usable shoots than observed in the first trial and in earlier work (Dinus et al. 1992a and b).

Considering all combinations of Z levels and subculture regimes, response to 1 mg/L Z and ASC56 (74.6 percent) significantly exceeded that of most other combinations. Response to 1 mg/L Z and ASC49 (41.9 percent), although not differing significantly from many other combinations, was roughly twice as large as any other response except that to 1 mg/L Z and ASC56 (74.6 percent), and 2 mg/L Z and CONTROL (38.1 percent).

The generally better performance of ASC and higher Z levels suggests that a relatively high but uniform Z level is important to continued explant health and supportive of shoot elongation. Poor response to NOGR combinations, regardless of initial Z level, supports such an argument. That is, more frequent subculture and/or higher initial Z levels seem to favor shoot production, whereas elimination of Z generally has an opposite effect. Absence of clear trends across Z levels within individual subculture regimes, possibly resulting from having only three replications, precluded drawing firm conclusions. As a result, another experiment was undertaken to confirm utility of ASC and evaluate need for additional measures to stimulate elongation.

In the third trial, K417 explants performed predictably, with callus and shoots forming soon after start of culture. Once again, K417 shoots elongated in large numbers throughout the test, regardless of GA treatment. GA was observed to increase shoot length, but effects on usable shoot numbers remained unclear. Both GA treatments produced more shoots than needed for other research, and definitive counts were not maintained. Observations also indicated that K417 shoots given GA were long and spindly. Harvest and transfer to rooting media were difficult. Further work is needed to gauge the nature of GA effects on such clones; lower GA concentrations deserve testing.

St75 shoots, as expected, began to elongate well after those of K417. Performance of ST75 nevertheless differed to some extent from that in the second trial; i.e., elongation occurred on smaller numbers of explants and somewhat sporadically across time, even though ASC56 had been implemented. Results agreed with earlier findings, however, in that ASC56 maintained explant health for a longer time. Indeed, explant condition was such that GA application to promote elongation was delayed beyond the time originally though appropriate for intervention (day 115 versus day 49 to 60). Within 21 days of application, shoots on explants given GA began to elongate, and usable shoots became available shortly thereafter. Explants given GA produced usable shoots

through day 203, a productive lifetime up to two times longer than those observed earlier (Table 1). In addition, numbers of usable shoots harvested across that time period were comparable to, or greater, than those produced by best treatments in most preceding trials.

Table 1: Summary of results for St75 from Trials 1-3 and a previous trial by Dinus et al. 1992a, b).

Trial	Treatments	Productive Explant Life (Days)	Usable Shoots/Explant (No.)
Previous	0.0 Z	91	0.00
	CIM4 \Rightarrow 0.5 Z	"	0.00
1	CIM4 \Rightarrow 0.5 Z	105	0.04
	CIM4 \Rightarrow 1.0 Z	119	0.14
2	CIM4 \Rightarrow 1.0 Z	126	0.00
	CIM4 \Rightarrow 1.0 Z + ASC56	161	1.85
3	CIM4 \Rightarrow 1.0 Z + ASC56	161	0.10
	CIM4 \Rightarrow 1.0 Z + ASC56 + GA	203	1.79

SUMMARY AND CONCLUSIONS

As apparent from Trials 1 and 2, moderate and relatively uniform Z levels seem more effective than other levels and monthly subculture at stimulating shoot production from internode explants of recalcitrant clones such as St75. Results from Trials 2 and 3 further indicate that ASC56 and 1 mg/L Z are beneficial to health of explants. The fact that the protocol fostered elongation only in Trial 2 infers that improved explant health may not be adequate for elongation and that some additional stimulus is required to obtain consistent results. GA application seems to be one approach to ensuring elongation, and more definitive research toward clarifying the contributions of ASC regimes and GA levels is underway. Shoots harvested from the present research were rooted at frequencies approaching 100 percent; regenerated plants are being maintained in culture and used as a source of explants for research on genetic transformation.

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UNDERSTANDING SUCROSE METABOLISM AND GROWTH IN A DEVELOPING SWEETGUM PLANTATION

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ABSTRACT

Stem diameter growth of 9-year-old sweetgum (Liquidambar styraciflua) trees was measured and related with the activity of sucrose synthase (SS), an enzyme that has been associated with carbon sink strength in agriculture crops and tree seedlings. In 1984, 1-0 sweetgum seedlings were transplanted to control plots and plots amended with sewage sludge or nitrogen and phosphorus fertilizer. After 8 growing seasons, trees on sludge contained four times as much volume as controls and twice as much volume as those on fertilized plots. In 1991, sludge treated soil had about 2 to 5 times as much in N and P as soils from other treatments. There were no differences in the K levels between treatments.

Beginning in 1992, sucrose metabolizing enzymes were assayed monthly in both phloem- and xylem-side stem cambial tissues. In late April when stem bark became easy to peel, there was SS activity in phloem-side but not in xylem-side stem cambial tissues. Phloem SS activity remained constant throughout the season. In less than 2 weeks, SS in xylem cambium exceeded that in phloem cambium by 20 folds and reached highest levels in June through August. For most of the growing season, there were no differences among treatments in the patterns for SS activity in xylem-side stem cambial tissues. However, SS activity in cambial tissues of trees from the sludge sites remained high in late September and October, when cambium of trees from the other treatments was dry and inactive and no SS activity was measured. Seasonal trends in activity of pyrophosphate-dependent phosphofructokinase (PPi-PFK) were similar to those of SS, but activity of its alternative enzyme, ATP-PFK, was low and constant throughout the growing season. We concluded that: (1) application of sewage sludge enhanced tree growth for at least 9 years, (2) sucrose metabolism is similar in 9-year-old sweetgum trees and in seedlings, and (3) sludge application increases the annual duration of high SS and PPi-PFK activities.

INTRODUCTION

All plant cells except green ones that are photoautotrophic, live off sucrose which is the major translocated carbohydrate in plants (Zimmerman and Brown 1971). Since sucrose is the starting point of glycolysis and the termination of gluconeogenesis, sucrose metabolism (sucrolysis) is essential for plants to grow, adapt to environmental changes, and survive stresses (Sung et al. 1988). Of three alternative enzymes catalyzing sucrose breakdown, sucrose synthase (SS) is the dominant activity in organs that are actively growing and storing reserves (Sung et al. 1989a; Xu et al. 1989). Acid invertase (AI) is closely associated with elongating tissues (Xu et al. 1989) whereas the role of neutral invertase (NI) is yet to be clarified. In nursery-grown sweetgum seedling taproots, SS activity decreased to minimum after leaf abscission in fall and did not rise again until June of the next year when leaves were fully expanded (Sung et al. 1989b). The periodicity of

growth in loblolly pine seedling stem and root was supported biochemically by high SS activity in stem cambium during summer and fall and in root cambium during fall and winter (Sung et al. 1993). Furthermore, SS activity declined during brief periods of low temperature and drought, when growth was suspended. Although the temporal (seasonal) and spatial (stem vs. root) patterns of sucrolysis in transplanted loblolly pine seedlings were not different from those of the nonlifted controls, SS activity of transplanted seedlings was much less than the controls (Sung et al. 1993). In fact, the duration and extent of transplanting stress was quantified biochemically via SS activity.

It has been suggested that there are two sets of enzymes in plant glycolytic pathways, namely the adaptive and the maintenance enzymes (Mustardy et al. 1986). The adaptive enzymes have large (5- to 10-fold) and rapid changes in activity in response to environmental changes and to plant growth and development. The maintenance enzymes usually have either low or very high levels of activity regardless the environments or plant status. Sucrose synthase, AI, and pyrophosphate-dependent phosphofructokinase (PPi-PFK) were identified as the adaptive enzymes in bean seeds (Xu et al. 1989), potato tubers (Sung et al. 1989a) and sweetgum and loblolly pine seedlings (Sung et al. 1989b; Sung et al. 1993). In the same plant tissues, NI and ATP-PFK were the maintenance enzymes that did not change their activities more than two folds. All these studies, however, were with an agriculture crop or 1- to 2-year-old seedlings. We report here a biochemical assessment of growth and stress in large plantation trees.

Recently, an 8-year-old sweetgum plantation established to determine the growth responses of three fertility treatments became available. Individual plots received sewage sludge, fertilizer, or no amendments. For 8 growing seasons, these initial fertility treatments have strongly influenced tree growth and vigor. The long duration of these growth differences suggested basic differences in sucrose metabolism enzyme activities among treatments. We therefore tested the following hypotheses: (1) developing plantation trees and seedlings have similar sucrose metabolism enzymes, (2) there are adaptive and maintenance enzymes in the glycolytic pathway in plantation trees, and (3) sucrose metabolism is more active or lasts longer in fast-growing, sludge-treated trees than in slower-growing trees.

MATERIALS AND METHODS

Site History

The original study evaluated the effects of subsoiling, sewage sludge, and vesicular-arbuscular mycorrhizae (VAM) on sweetgum growth on high-quality soil located at the Savannah River Site, Aiken, SC. The experimental design was a split-split plot with four replicate blocks. Treatment variables were fertility, subsoiling, and VAM. In July 1983, sewage sludge from Athens, GA, was applied to one-third of each block at 34 dry metric tons/ha and then all plots were double disked. Another one-third of each block received fertilization treatment consisted of 280 kg/ha diammonium phosphate in 1985 and 240 kg/ha ammonium nitrate in 1986. The remaining one-third of each block received no amendments and served as the control. In September 1983, half of each block was subsoiled with furrows in parallel lines 122 cm apart and 76 cm deep. Sweetgum seedlings were produced at Whitehall Experimental Nursery in

Athens, GA. VAM seedlings had been inoculated with Glomus fasciculatum or G. macrocarpus. Both inoculated and noninoculated seedlings were grown in fumigated nursery beds with 75 to 100 ppm P. Sixteen seedlings with 6 or more first-order lateral roots were planted at a 3 x 3 m spacing in each plot in February 1984.

Tissue sampling and preparation

For the purpose of current study, there were three soil fertility treatments: sludge, fertilizer, and control. Cambium samples were taken monthly in 1992; and the same protocol will be continued in 1993. Two to three strips of 3.0 x 20 cm stem bark were removed from each tree at breast height (1.5 m aboveground). For each treatment, two trees from each of the two blocks were sampled in 1992. In 1993, dendrobands were put on the interior trees in the other two blocks. These trees will be sampled for enzyme activity and stem diameter growth. Phloem cambial tissues were scraped from the inside of the bark and xylem cambial tissues were scraped from the exposed surface of the stem. The scraped tissues were immediately frozen in liquid nitrogen and transported back to the lab. The next day tissues were homogenized for enzyme assays. The enzyme extraction and assay procedures followed Sung et al. (1993). Activities of sucrose synthase (SS), acid invertase (AI), neutral invertase (NI), pyrophosphate-dependent phosphofructokinase (PPi-PFK), and ATP-PFK were routinely assayed from the same extracts.

RESULTS AND DISCUSSION

In 1991, the eighth growing season after planting sweetgum plantation, leaves of trees grown in the sludge plots were retained and were functionally green until the frost late in November. Leaves of tree in the other plots became discolored in mid-September and fell soon after. In addition, all the trees in the sludge plots had bumper seed crop while other trees produced few if any seeds. In 1991 stems were measured. Since no important effects of the VA or subsoiling treatments were observed, data were compiled into three groups based on the initial fertility treatments - sludge, fertilizer, and control. Trees grown on sludge contained 4 times the volume of control trees and twice the volume of fertilizer trees. Trees on sludge were taller and larger in diameter at breast height (DBH) than the others. Trees grown on the fertilizer plots were taller and larger in DBH than control trees. It was reported that 5 years after sewage sludge treatment applied at pole-stage, sawlog volume increased 50% in Corsican pine (Pinus nigra var. maritima (Ait.) Melville) (Moffat et al. 1991).

In addition to measuring tree growth in 1991, we collected soil samples from each plot at two depths, 0 to 7.5 and 7.5 to 15 cm for soil nutrient analyses. The average concentrations of major element levels for the three fertility treatments were:

Treatment and depth	Concentration (ppm)		
	N	P	K
Control			
0-7.5	370	29	29
7.5-15	267	24	24
Fertilizer			
0-7.5	389	36	25
7.5-15	289	28	23
Sludge			
0-7.5	1153	153	24
7.5-15	448	143	24

Eight years after treatment sludge plots contain 2- to 5-times as much total N and available P as the other soils. No differences in soil fertility level were found between soils of control and fertilizer treatments. Generally, there was more N in the top 7.5 cm soil than the next soil layer in all three treatments. In a study by Berry and Marx (1980), 3 years after the sludge treatment, there were 595 and 84 ppm of N and P in the soils whereas the control soils had 112 and 7 ppm on N and P, respectively.

Based on the 1991 soil fertility analysis the growth advantage for trees on sludge plots seems likely to continue for a few more years. In 1992, DBH increased 4.3, 2.1, and 1.1 cm, respectively, for trees in sludge, fertilizer, and control plots. Average increases in DBH among trees sampled for enzyme activity were 1.44, 0.99, and 0.97 cm during 1992. Reasons for the slower growth of sample trees are not clear. Bark strip removal may be the cause. Trees sampled before July produced some callus tissues along the cut edges, whereas no callus formation was noticed after June sampling.

As in sweetgum and loblolly pine seedlings (Sung et al. 1989b; Sung et al. 1993), sucrolysis with sucrose synthase was the dominant sucrose breakdown activity in the 9-year-old plantation sweetgum. No clear seasonal patterns in AI or NI activity were observed (data not shown). SS activity in phloem-side stem cambial tissues ranged from 5 to 35 nmol per mg protein per min throughout the sampling periods. In the first sampling in April 1992, no SS activity was detected in xylem-side cambium. The data suggested that phloem cambium became active earlier than xylem cambium in spring (Figure 1). Within 2 weeks, xylem cambium became active, and its SS activity was 10 to 20 times higher than that in than phloem throughout the year. On a fresh weight basis, the xylem-side cambium has 4- to 10-fold as much soluble protein as that of phloem-side cambium (data not shown). Loblolly pine seedling stem phloem-side cambium also had low soluble protein and SS activity (Sung et al. 1993). It is generally acknowledged that for every cell division in phloem mother cell region there is at least 6 or 7 cell divisions taking place in xylem mother cell region.

Xylem cambium became active about 1 week earlier in 1993 than in 1992 (Figure 1). Except toward the end of the growing season, there were no differences in SS activity between treatments. From June to August, SS activity was highest in all trees. SS activity, however, remained high for at least 1 more month in sludge-treated trees than other trees (Figure 1). Therefore, an extended duration of high SS activity rather than high absolute activity gave sludge trees their growth advantage. Since the active cambial tissues were most scrapable, one needs to consider whether there were any differences in SS activity per unit of cambial area.² Basically, the trends did not change when SS activity was expressed in nmol/cm².min (Figure 2) as in Figure 1. In both Figure 1 and 2, there was a second peak in SS activity of sludge trees in September. The functionally green leaves on trees of sludge plots in September certain can export sucrose for stem cambial SS to metabolize it for growth. The continuous declining SS activity in other trees in fall coincided with leaf discoloring and abscission.

SS activity was also assayed monthly in xylem-side cambial tissues of lateral roots. However, the variations within treatments were too large to draw meaningful conclusions. In fact, within the same piece of lateral root there were differences in the ease of bark peeling. In July and August, root SS activity averaged about 50 nmol/mg protein.min for all treatments. This rate was much lower than that of the seedling taproot which is the major belowground sink for sucrose (Sung et al. 1989b). A plantation tree has many lateral roots which probably are not equally competitive sucrose sinks at the same time.

As in earlier studies with seedlings (Sung et al. 1989a; 1989b; 1993), the PPI-PFK activity in plantation trees was that of an adaptive enzyme; it correlated well with growing season (Figure 3). Furthermore, the similarity between seasonal patterns of PPI-PFK and SS in loblolly pine seedlings (Sung et al. 1993) was observed with sweetgum trees (Figure 2 and 3). ATP-PFK activity, on the contrary, was that of a maintenance enzyme. Like the invertases, ATP-PFK was more or less constant in activity throughout the year (Figure 4).

We concluded that sucrolysis in sweetgum plantation trees is similar to that in nursery seedlings. Throughout the growing season, SS is the dominant sucrose breakdown activity and it correlates well with physiological activity. Trees on sludge-treated soil grew more in diameter and had high SS and PPI-PFK activities for a longer-period than control and fertilized trees. The long-term positive effects of sludge on sweetgum tree growth and seed production are especially important for tree improvement. Monitoring of SS activity increases our understanding of the biochemical basis for the growth advantage.

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FIGURE LEGENDS

Figure 1. Seasonal changes in sucrose synthase specific activity, nmol/mg protein.min, in stem xylem cambial tissues of plantation sweetgum trees. Each data point was the average of four samples. The same plant extract was used for all enzyme assays.

Figure 2. Seasonal changes in sucrose synthase specific activity, nmol/cm².min, in stem xylem cambial tissues of plantation sweetgum trees. Data from Figure 1 was used to calculate these values.

Figure 3. Seasonal changes in P_{Pi}-dependent phosphofructokinase specific activity, nmol/cm².min, in stem xylem cambial tissues of plantation sweetgum trees. Each data point was the average of four samples.

Figure 4. Seasonal changes in ATP-dependent phosphofructokinase specific activity, nmol/cm².min, in stem xylem cambial tissues of plantation sweetgum trees. Each data point was the average of four samples.

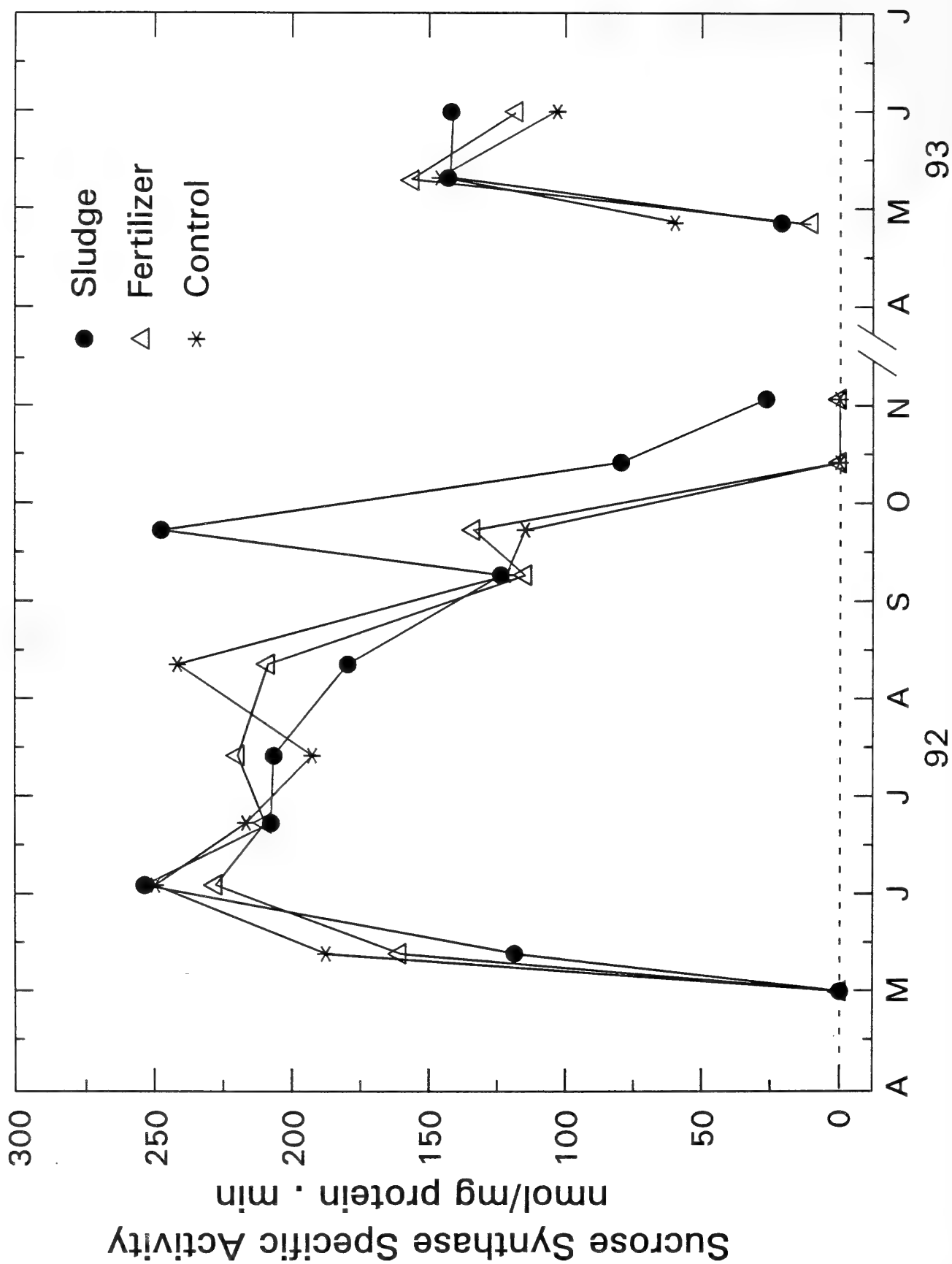


Figure 1

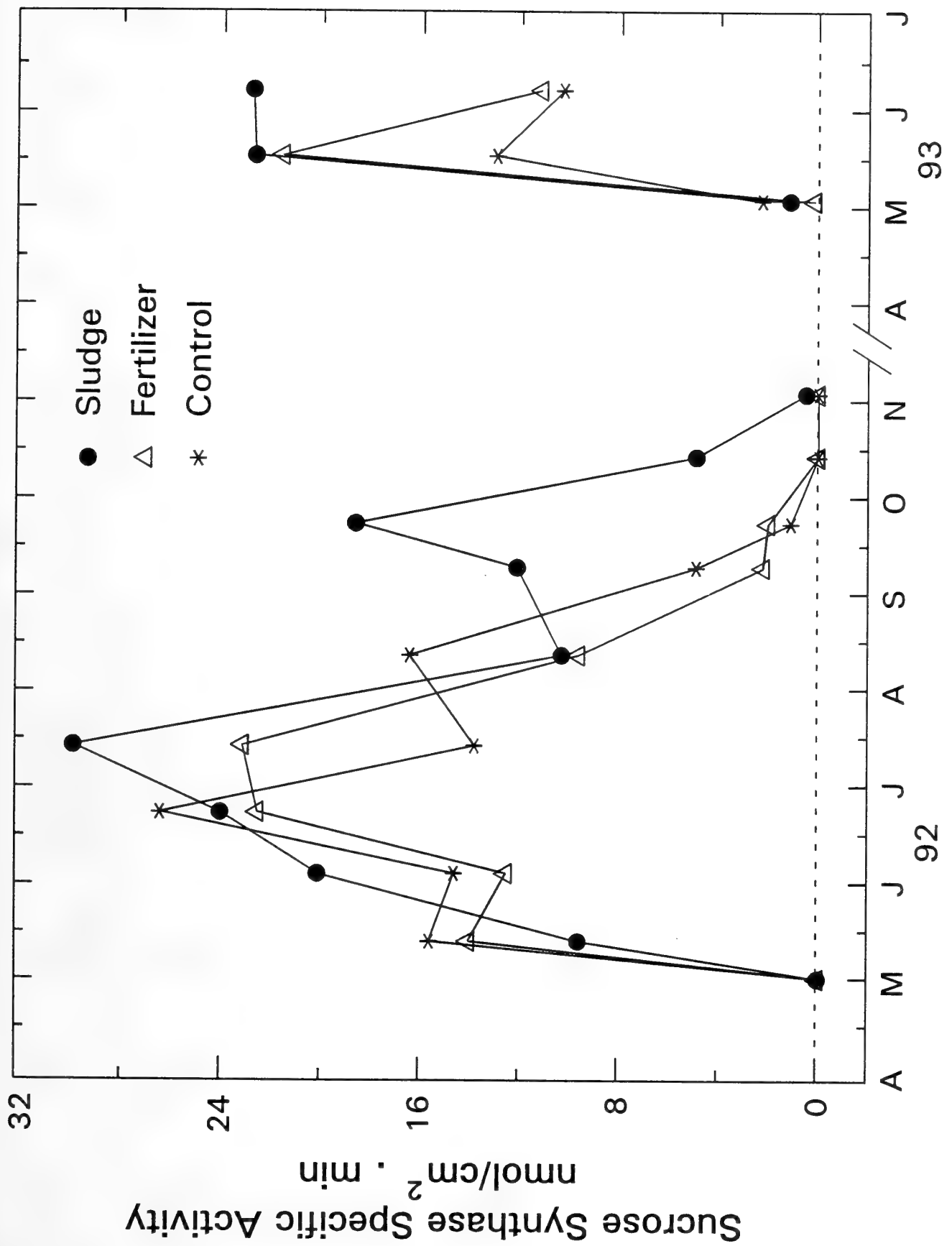


Figure 2

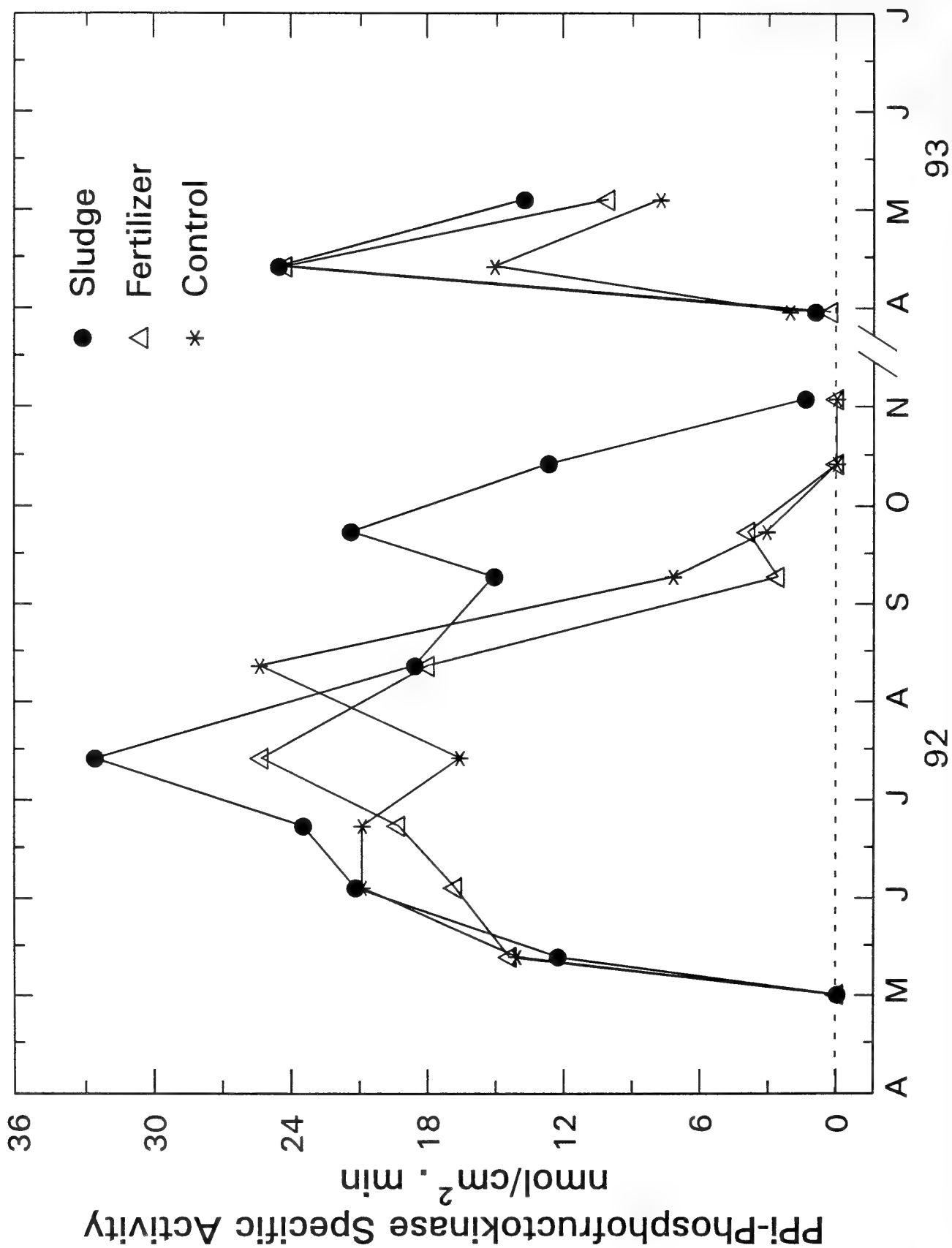
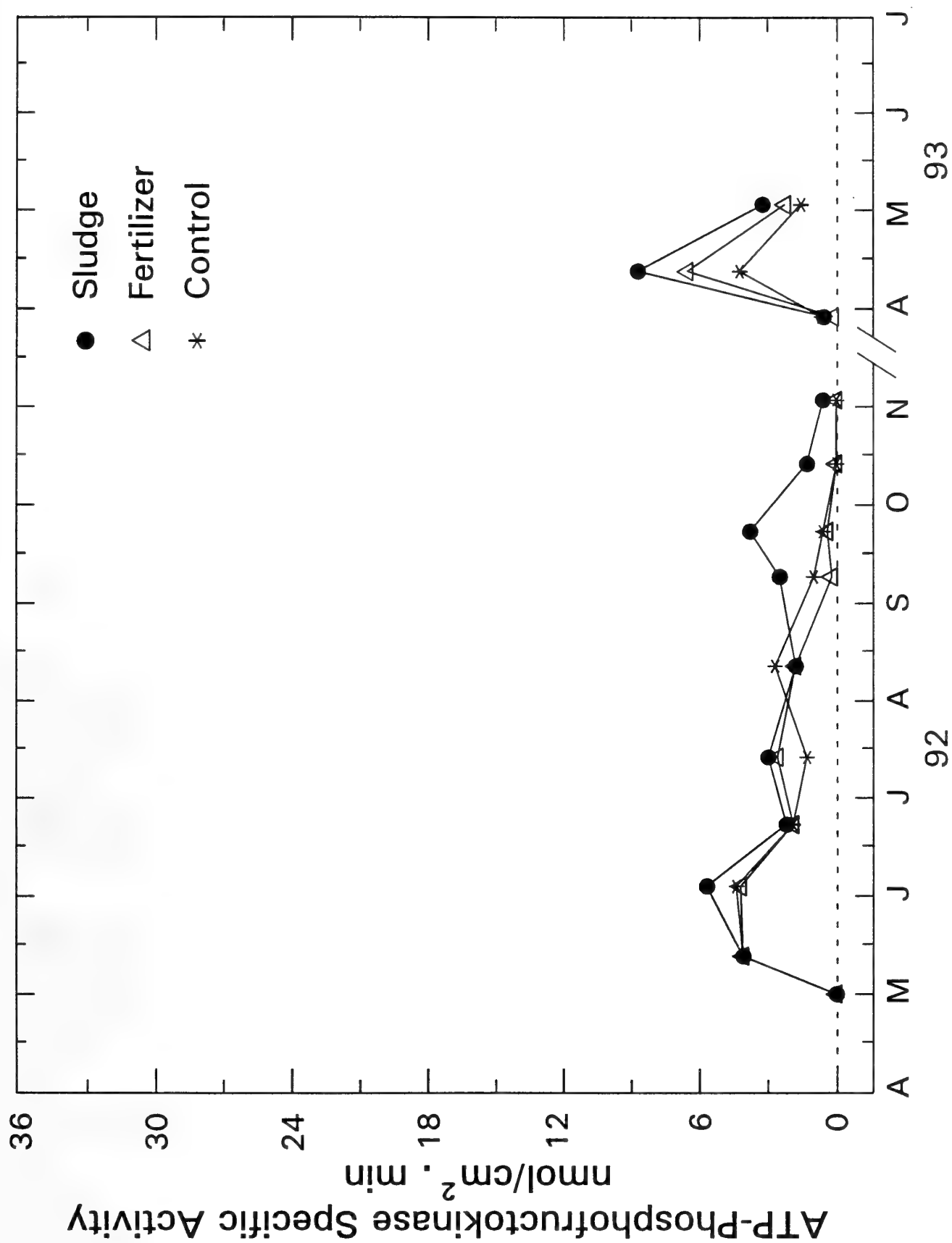


Figure 3



SELECTION OF SWEETGUM AND SYCAMORE FROM PROVENANCE-PROGENY TESTS

B. C. Bongarten and J. Liang¹

Abstract. In support of the N.C. State Cooperative Hardwood Research Program plan to establish seed orchards of improved Southern hardwoods, scions were collected from selected trees in a 13-year-old sweetgum provenance-progeny test and a 10-year-old sycamore provenance-progeny test, both established in the Georgia Piedmont. The primary basis for tree selection was expected breeding value for volume. Expected breeding values were determined for each test tree from indices incorporating provenance, family within provenance, plot within family or provenance, and tree within plot values. A spline regression model was used to describe and eliminate environmental variation in the tests, markedly improving heritabilities. Indices were constructed using log transformed data to minimize effects of site quality on selection. Provenance means had the greatest influence on breeding values. Family within provenance and tree within plot were next in importance and of equal influence. Plot within family or provenance had a negligible influence. In making final selections, consideration was given to maximizing family diversity and stem straightness.

Keywords: *Liquidambar styraciflua*, *Platanus occidentalis*, selection indices, progeny tests, advanced generation selection.

INTRODUCTION

This year, the North Carolina State Cooperative Hardwood Research Program assembled selections from progeny tests of several Southern hardwood species for later incorporation in seed orchards. We, at The University of Georgia, contributed sweetgum (*Liquidambar styraciflua*) and sycamore (*Platanus occidentalis*) selections to that collection. In this paper, we describe what selections were made, and how they were chosen.

For both sweetgum and sycamore, selections were made from provenance- progeny tests established more than 10 years ago. The primary criterion for selection was predicted breeding value for current age volume, but consideration was also given to form characteristics and to maintaining genetic diversity.

In constructing the indices for breeding value determination, three practices were employed which may not be considered standard. First, environmental trends within the progeny test were modelled with spline regression and then eliminated. Second, the within family term in the selection indices was divided into plot within family and within plot components. Third, the indices were build with log-transformed data.

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PROGENY TEST DESCRIPTIONS

Sweetgum

Sweetgum selections were taken from a single provenance-progeny test established in February, 1980 with 1+0 stock grown in a nursery at The University of Georgia. Seeds for that test were provided by K. C. Steiner of The Pennsylvania State University and by the senior author. Fifth-year results of this and related tests were reported in an earlier SFTIC Proceedings (Steiner *et al.* 1985).

The test contains trees from four (or rarely three) open-pollinated families from each of 27 provenances located throughout the natural range of sweetgum east of the Mississippi River. The test design is split-plot with four single-tree family subplots within each provenance main plot, replicated in four complete blocks. Tree spacing was 2.6 x 2.6 m. Survival was 95% leaving 410 test trees for consideration.

The test site is located about ten miles north of Eatonton, Georgia, in Putnam County, in the lower Piedmont. The soil at the site is an eroded Cecil, typical of Piedmont uplands. Average tree height after thirteen field seasons was approximately 6 m.

Sycamore

Sycamore selections were also taken from a single provenance-progeny test. This test was established in March, 1982 with 1+0 stock grown at The University of Georgia. The test contains trees from three (occasionally two) open-pollinated families from each of 80 provenances located throughout the native range in Georgia and adjacent parts of South Carolina, Alabama and Florida.

The test design is a randomized complete block with two-tree family plots established in each of four replications. Tree spacing was 3 x 3 m. Altogether, 1724 trees from 221 families were planted. The survival was 91%, leaving 1563 trees for consideration.

This test is located about one mile from the above-mentioned sweetgum test, but on a bottomland site (bordering the Little River). The soil at this location was typed as a mix of Chewacla and Starr series soils. Average height of dominant and co-dominant trees after ten field seasons was approximately 14 m, with the largest trees over 20 m.

PREDICTING BREEDING VALUES

Subtracting Environmental Variance

If one can reduce environmental variation in a progeny test, the effectiveness of selection can be improved by increasing the heritability. One practice that has been productive for this purpose is subtracting the block effect from individual phenotypes before analysis (Cotterill 1987). The intent of this procedure is to remove variation associated with environmental trends within the progeny test.

An elaboration of this technique is to use spline regression, rather than blocks, to describe environmental trends (Bongarten and Dowd 1987). The spline regression method allows for

modelling of complex trends, and may, in many circumstances, account for more environmental variation than blocks.

To describe environmental trends with spline regression, the genetic values of the data must be spatially randomly distributed. Therefore, when the test design includes contiguous-tree plots, it may only be applied to plot means. In the case of the sweetgum test, a spline regression was fit to the provenance plot means. For the sycamore test, it was fit to family plot means.

The effectiveness of spline regressions in reducing environmental variation and increasing heritability may be seen in Figure 1. There, heritability for relative volume (D^2H) after subtraction of block effects is compared with heritability for relative volume after subtraction of environmental effects determined by spline regression. For sycamore, individual tree heritability increased from 0.27 to 0.37 when spline regression was used in place of block adjustment. A similar comparison could not be made for sweetgum because individual tree heritability was calculated from within provenance plot deviations which were not affected by either adjustment technique. However, provenance mean heritability increased from 0.85 to 0.95 when spline regression was used. As a consequence of these results, the selection indices described below were constructed using deviations from the values predicted by spline regression as data.

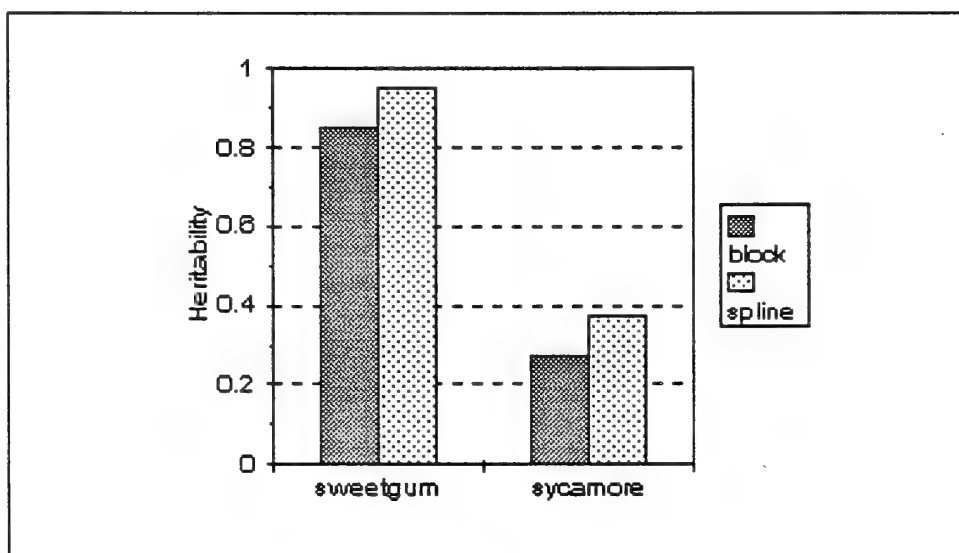


Figure 1. Provenance mean heritability in the sweetgum progeny test and individual tree within provenance heritability in the sycamore progeny test following subtraction of environmental effects determined by block means and spline regression.

Development of Selection Indices

The purpose of selection indices in the present case is to determine expected breeding values of progeny test individuals for current-age volume. The indices incorporate information from each observational effect contributing to the individual's phenotype following Land *et al.*'s (1987) approach. The observational effects are also called "sources of variation" in an ANOVA table (see Table 1).

Provided that each of the observational effects is independent of the others, the breeding value (A_i) of an individual may be estimated as the sum of the additive genetic effects contributed by each effect (Falconer 1991):

$$A_i = \sum A_{xi} \text{ over all } x\text{'s} \quad (1)$$

where x 's are the observational effects and A_{xi} = the additive genetic effect of observational component x on individual i . Each A_{xi} may be estimated by:

$$A_{xi} = sd_{xi} \cdot h_{xi}^2 \quad (2)$$

where sd_{xi} = the value of observational effect x for individual i , and h_{xi}^2 is the portion of the variation in observational effect x which is additive genetic; i.e., each observational effect represents a selection differential, which may be multiplied by an appropriate heritability to yield the portion which is additive genetic.

In developing the heritabilities for the observational effects, it was assumed that all genetic variation among provenances was additive. That is, crosses between provenances would yield progeny that were exactly intermediate between the parents. It was also assumed that the coefficient of genetic relatedness (r) for trees within a provenance was 0.30. In all cases, family effects were treated as family within provenance effects.

Sweetgum: For sweetgum, individual phenotypes (P_i) may be considered to be the sum of the following observational effects, expressed as deviations from the test mean: provenance (Pr), plot within provenance (Pl), family within plot within provenance (F) and within family between plots (W) (Table 1). Therefore, for each individual, i ,

$$P_i = Pr_i + Pl_i + F_i + W_i \quad (3)$$

Note, the total within family variation is divided between two components -- and among plot component, Pl, and a within plot component, W. The among plot component is due to the fact that, by chance alone, a plot may be composed of trees with above (or below) average breeding values.

Sycamore: For sycamore, the observational effects that make up an individual's phenotypic effect are somewhat different because of the different experimental design (Table 1). However, the within family variation is still divided between an among plot component and a within plot component.

Scaling Data

In progeny tests, the phenotypic variance of growth traits typically varies with tree size and site quality. Generally, variance increases as tree size or site quality increases, at least during the site-capture years. As a result, if site quality varies within the test, most of the selections will come from the high quality sites when absolute measures of tree size serve as selection criteria.

The problem may be solved by scaling growth measures to units whose variance is not related to tree size. Log-transformation is often applied in such cases. When applied to the sweetgum and sycamore progeny tests under consideration, it had little influence on family ranking, but it did alter breeding values within families so that they were more randomly

distributed throughout the test. Therefore, we based selections on estimates of breeding values of log-transformed data.

IMPORTANCE OF PROVENANCE, FAMILY AND WITHIN FAMILY VARIATION

The mean of the expected breeding values of the selected trees is the expected gain made from selection. And, the mean of the breeding values may be viewed as the sum of the gains contributed by each component in the selection index (equation 1). Because the gain from each component may be calculated as the selection differential times the heritability (equation 2), and the intensity of selection is approximately equal for each component, the relative gain from each component may be determined as the product of the phenotypic standard deviation of the component and the heritability of the component.

Table 1. Relative gain contributed by provenance, family and within family variation as determined by the product of the phenotypic standard deviation and the heritability of each source of variation.

SWEETGUM		SYCAMORE	
Source of Variation	Relative Gain (%)	Source of Variation	Relative Gain (%)
Provenance	55	Provenance	60
Family w/in Prov.	17	Family w/in Prov.	20
Plot w/in Prov.	6	Plot w/in Family	0
w/in Family w/in Plot	22	Tree w/in Plot	20

For both sweetgum and sycamore, the largest share of the gain (about 60%) comes from provenance selection (Table 1). This is to be expected in sweetgum where the provenances cover a large geographic range, and there is a strong tendency for trees from north of 35° N latitude to be smaller than those from farther south (Figure 2). For sycamore, the great gains from provenance selection may result because trees within a provenance have common parentage; gene flow for species distributed linearly, as along rivers, is expected to be less than for species whose coverage is wider (Wright 1978).

Forty percent of the gain, in both species, comes from selection within provenances. This is distributed approximately equally between gains from family and within family selection (gains from within family selection may be calculated as the sum of among and within plot terms). Although family (within provenance) heritability was greater than within family heritabilities, over two-thirds of the additive genetic variation within a provenance lies within a single open-pollinated family.

If one divides total gain into family and within family components, then the family component, comprised of the provenance and family within provenance sources, accounts for approximately three-quarters of the total gain; the within family component makes up the remaining one-quarter.

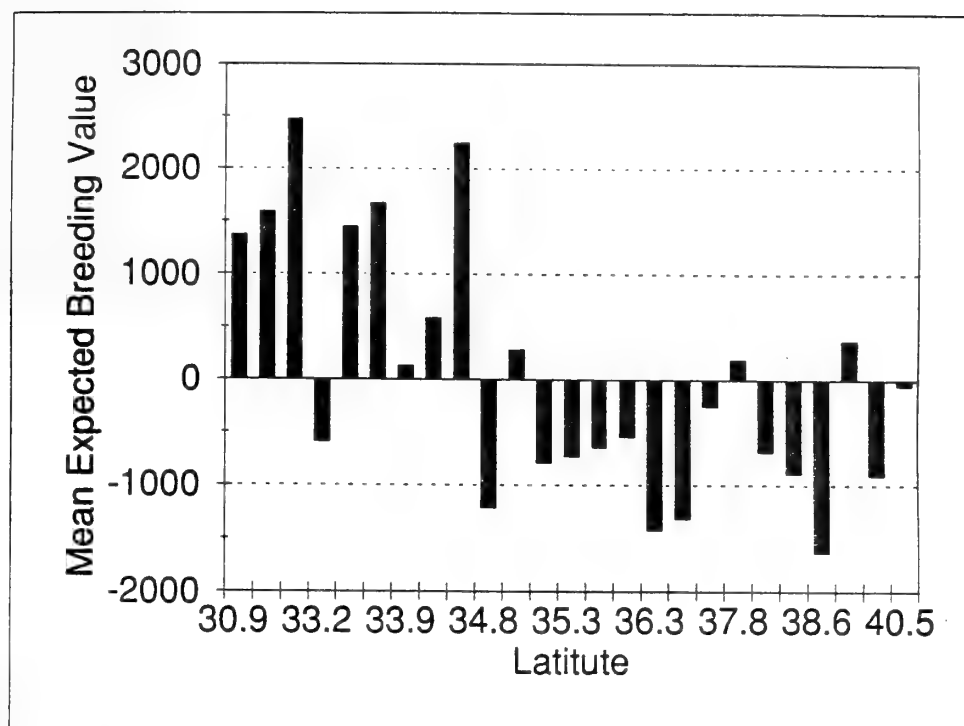


Figure 2. Mean expected breeding values for sweetgum provenances arranged by latitude of origin.

CONCORDANCE OF SELECTION FOR HEIGHT, DIAMETER OR VOLUME

In addition to estimating breeding values for volume, we also estimated breeding values for height and diameter. The same procedures used to estimate volume breeding values were employed to estimate height and diameter breeding values. Although many trees having high expected breeding values for volume also had high expected breeding values for height and diameter, this was not always the case. Table 2 shows the ten sycamore families with the highest mean breeding values for volume. Of these, seven were also among the top ten for height, and six were among the top ten for diameter. Only four families were in the top ten for height and diameter. Therefore, selection based on height or diameter would produce a different population than selection based on volume, even though height and diameter are strongly correlated with volume. Furthermore, these results provide evidence for genetic differences in height:diameter ratio -- differences which are visually evident in walking through provenance tests of either species.

SELECTION CRITERIA

As previously mentioned, in assembling the selected population, the principle selection criterion was volume breeding value, estimated by the selection index. However, several other criteria were also used to some extent in the final selection. (1) **Genetic diversity:** Because of the large family component to gain, the best families had several members with high expected breeding values. In most cases we decided to include no more than one tree per family in the selected population to maintain genetic diversity, allowing users of the material flexibility to

design seed orchards for varying needs. (2) **Form:** Most sweetgums and sycamores have very straight stems. However, some fast-growing trees exhibit sinuosity. Trees with sinuous stems were not selected. (3) **Sufficiency of collectable material:** In a few cases we were not able to collect ten usable scions from a tree selected on the basis of volume breeding value. In such cases, the tree was not utilized.

Table 2. The top ten sycamore families for volume breeding value (ranked in descending order) and their presence (✓) or absence (—) in the group of ten families having the highest height or diameter breeding values.

	TRAIT		
	Volume	Height	Diam.
332	✓	✓	✓
122	✓	✓	✓
120	✓	—	—
182	✓	✓	✓
167	✓	✓	✓
151	—	—	—
145	✓	—	—
179	✓	—	—
134	—	✓	✓
567	—	✓	✓

DESCRIPTION OF THE SELECTED POPULATIONS

Of the 410 sweetgums available for consideration, 14 were selected. Scions of these were forwarded to North Carolina State University for inclusion in a clone bank. Of the 1563 sycamores available for consideration, 11 were selected. Scions of these were forwarded to Union Camp Corporation for inclusion in a clone bank. Family numbers and origins of the selected trees are shown in Table 3. Most selections are from trees originating in the Coastal Plain; only sweetgum families 11, 13, 14 and 66 and sycamore family 565, all from the Piedmont, are not.

While collecting scions from the selected trees, in early March, 1993, we observed considerable variation in traits which were not used as selection criteria including, height:diameter ratio, crown width, branch thickness, frequency of branches along the bole, branch angle, presence of lammass growth, autumn frost damage and time of spring bud swell. These traits appeared to be relatively consistent within a family, suggesting a genetic component to their expression. Our impression is that much of the variation we observed in these traits is included within the population of selected trees. This should help to provide breeders the flexibility to develop custom varieties to meet varying needs in the future.

Table 3. Family and geographic origin of the selected sweetgums and sycamores.

SWEETGUM		SYCAMORE	
UGA Family No.	County, State of Origin	UGA Family No.	County, State of Origin
1	Burke, GA	120	Burke, GA
2*	"	121	"
11	Greene, GA	122	"
13	"	134	Jackson, FL
14	"	145	Dougherty, GA
16	George, MS	146	"
27	Jones, NC	167	Treutlen, GA
28	"	182	Upson, GA
29	"	332	Laurens, GA
33	Covington, AL	565	Taliaferro, GA
34	"	567	Jefferson, GA
66	Greenwood, SC		
90	Lancaster, VA		

*Two selections were chosen from this family. In all other cases only one tree per family was chosen.

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SESSION 3

Softwood Propagation

ABSCISIC ACID LEVELS IN EMBRYOS AND MEGAGAMETOPHYTES OF *PINUS TAEDA* L.

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Abstract. -- Absciscic acid (ABA) was quantified in developing zygotic embryos and megagametophytes of loblolly pine using indirect ELISA. On a per tissue basis, the amount of ABA present in the megagametophyte remained relatively constant at approximately 2.5 ng/megagametophyte. The ABA in embryos remained low during early to mid development, peaked to approximately 1.5 ng/embryo, before declining and peaking a second time prior to cone ripening. On a g dry weight basis, ABA in megagametophytes exhibited a steady drop from approximately 1750 ng in early development to 127 ng at cone ripening. ABA in embryos was high during early development (2250 ng/g dry weight) and displayed two significant peaks middle to late development before dropping to approximately 600 ng/g dry weight at cone ripening. Peak levels of ABA in embryos (on a tissue basis) appear to occur shortly before the increase in dry weight accumulation.

Key words: ELISA, loblolly pine, zygotic embryogenesis, somatic embryogenesis, seed maturation.

INTRODUCTION

Endogenous absciscic acid (ABA) has been implicated as a factor involved in regulating seed development and maturation. Putative roles of ABA in seeds during embryogenesis include inhibition of precocious germination, promotion of storage protein synthesis, suppression of reserve mobilization, promotion of desiccation tolerance, and induction of dormancy (Black 1991). In conifer somatic embryogenesis, exogenous ABA shows similar critical functions: inhibition of precocious germination and accumulation of storage nutrients, lipids, and proteins in embryos cultured on media containing specific levels of ABA in *Picea glauca* (Attree et al. 1992), *Picea glauca engelmanni* (Roberts 1991), *Picea abies* (Hakman et al. 1990), and *Pinus taeda* (Becwar and Feirer 1989). Enhancing lipid biosynthesis using ABA and polyethylene glycol improved desiccation tolerance in somatic embryos of *Picea glauca* (Attree et al. 1992).

Although ABA plays a critical role in the development of conifer zygotic and somatic embryos, very little is known about the biochemical and molecular events that occur during conifer zygotic embryogenesis. This incomplete knowledge makes the development of somatic embryogenesis difficult, particularly for loblolly pine, which has proven difficult to manipulate in culture. Usable embryos and seedlings have been produced, but successes remain sporadic and highly dependent on genotype (Gupta and Durzan 1987). Trial and error experimentation has been the hallmark of research on this technology, making it time-consuming and labor-intensive efforts.

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The objective of our research has been to use zygotic embryogenesis as a model to determine optimal ABA requirements for the somatic system. Concentration changes of ABA were measured in embryo, megagametophyte, and suspensor tissues during zygotic development. These trends will be used to adjust those used for somatic embryogenesis. Using the zygotic system as a model should make research to develop somatic embryogenesis more effective and efficient.

MATERIAL AND METHODS

Chemicals and Supplies. Buffers. PBS/Tween: 13.6 g KH_2PO_4 , 29.2 g NaCl per liter containing 0.05% Tween 20 (buffer adjusted to pH 7.4); blocking solution: 5% (w/v) bovine serum albumin (BSA) (Sigma, A-3425) in PBS/Tween; assay buffer: 0.1% (v/v) BSA in PBS/Tween. ABA-4'-BSA Conjugate. The conjugate was prepared according to Quarrie and Galfre (1985) and stored at -20°C in its concentrated form. The conjugate was diluted in 0.05 M NaHCO_3 (pH 9.6) to a concentration (1:100,000) determined by optimization trials. Monoclonal Antibody. The monoclonal antibody (mAb) raised against free *cis*-(+)-ABA (Mertens et al. 1983) was from Idetek, Inc., San Bruno, CA. The mAb was dissolved in 2 mL 0.1% BSA, aliquoted into 10 μL portions and stored at -80°C . The monoclonal was further diluted in assay buffer to a concentration (1:5000) as determined by optimization trials. Goat Anti-mouse Antibody-Biotin Conjugate. Goat anti-mouse antibody-biotin conjugate (Sigma, B-7264) was aliquoted into 10 μL portions and stored at -20°C . The conjugate was diluted 1:5000 in assay buffer prior to use. Streptavidin-Poly-HRP Conjugate. Streptavidin-Poly-HRP (Research Diagnostics, Inc., Flanders, NJ, No. RDI-pHRP20-SA) was aliquoted into 10 μL portions and stored at -20°C . The conjugate was diluted 1:5000 in assay buffer prior to use. Tetramethylbenzidine (TMB) Substrate. A 10 mg/mL solution of TMB (Sigma, T-2885) in DMSO was diluted 1:1000 in 100 mM $\text{Na}_2\text{H}_2\text{O}_2$ (pH 5.5). Hydrogen peroxide (3% solution) was added to a final concentration of 0.002%. Abscisic Acid (ABA) Standards. A stock solution of 50 mM (+)-*cis*-ABA was prepared by dissolving (+)-*cis*,*trans*-ABA (Sigma, A-1012) in absolute methanol. This solution was stable for at least three months when stored at -20°C and darkness. Standards ranging from 15.8 ng to 0.25 pg/100 μL (+)-ABA were made in assay buffer by serial dilution. The small amount of methanol in the standards did not affect the performance of the antibody (data not presented). Microplates. Immulon-2, flat-bottom, 96-well microtitration plates (Dynatech Laboratories, Chantilly, VA) were utilized for optimal binding of the ABA-4'-BSA conjugate (Walker-Simmons 1987). Microplate Reader and Washer. Absorbencies were read using a Bio-Tek Kinetic Reader Model EL312E at 630 nm and 450 nm wavelengths. Micro-plates were aspirated and washed using a Bio-Tek manual washer Model EL40112.

Plant Material. Loblolly pine cones from four seed orchards (generously provided by Westvaco Corp., Weyerhaeuser Co., and Union Camp Corp.) were shipped overnight, on ice packs, on a weekly basis from June to October 1992 (fertilization to cone ripening). Clones were designated "WV" for Westvaco, "WA" and "WB" for Weyerhaeuser, and "UC" for Union Camp. Seeds were immediately extracted from the cones upon delivery, and embryos, suspensors, and megagametophytes were separated under a stereoscopic microscope. The embryos were staged for morphological development and briefly rinsed in distilled water to remove extraneous hormones that may have leaked from the megagametophyte during the dissection. The three tissues were frozen separately in liquid nitrogen, and the collected material was then lyophilized and stored at -80°C until analyzed. Tissues were thus stored based on clone, tissue type, and stage of development.

Staging of Developing Embryos. Staging of the tissues was based on morphological changes of the developing embryos using a method developed at IPST (Webb 1991). Stage 1: proembryos, from free nucleate to 12 cells; Stage 2: embryo proper, microscopic but distinct/translucent, still found at micropylar end; Stage 3: embryo proper becomes white/opaque and is found at chalazal end; Stage 4: larger than Stage 3, opaque embryo proper enlarged longitudinally, dome shaped apex; Stage 5: similar to Stage 4, except apical meristem is visible; Stage 6: similar to Stage 5, except cotyledon primordia are barely visible below apical meristem; Stage 7: similar to Stage 6, except cotyledons are elongated, but do not overtop apical meristem; Stage 8/8B: similar to Stage 7, except cotyledons overtop apical meristem, apical meristem still visible; Stage 9: cotyledons curved and joined at their tips; apical meristem is not visible. Stage 9 tissue was subdivided by percent moisture content of tissue since growth continued without further obvious morphological change (9A-9I).

ABA Analysis. ABA Extraction. Isolated tissues were ground using a cold glass rod, weighed, and extracted in 80% methanol (v/v) containing 25 mg/L butylated hydroxytoluene (BHT) adjusted to pH 7. Approximately 10^5 DPM of [3 H]-ABA (Amersham, Arlington Heights, IL, TRK.644) was added as an internal standard. Tissues were extracted overnight with stirring at 4°C in the dark.

ABA Purification. The homogenate was centrifuged at 2000 g for 15 minutes and the supernatant removed. The pellet was resuspended in 1 mL of extracting solvent and recentrifuged at 2000 g for 10 minutes. The supernatants were pooled, passed through a 0.45 μ m nylon filter, and reduced to near dryness using rotoevaporation in vacuo at 35°C and dim lighting. The remaining aqueous phase was diluted to 1000 μ L in assay buffer for analysis by indirect ELISA. This procedure gave an optimum extraction of ABA of 92% (95% CI 2.5%) recovery.

ABA Quantification. This procedure is modified from Walker-Simmons (1987) by addition of a biotin-streptavidin-multiple horseradish peroxidase (HRP) system for amplification. **Coating of microtiter plate.** Diluted ABA-4'-BSA conjugate (200 μ L) were added to each well of the microtiter plate except those in the outside rows as they have been shown to produce inconsistent results (Ross et al. 1987). The plates were sealed with Parafilm and incubated overnight at 4°C in the dark. **Incubation of standards or samples with ABA antibody.** ABA standards and samples were diluted 1:1 with the diluted monoclonal antibody and incubated overnight at 4°C in the dark. Triplicate standard curves were included on every plate. Samples were diluted in assay buffer to obtain absorbencies in the center of the calibration curve. **Blocking of the wells.** Plates were aspirated and washed four times with PBS/Tween. Approximately 300 μ L of blocking solution were added to each well, and left to incubate for 45 minutes at 37°C in the dark. The wells were aspirated and washed four times with PBS/Tween. **Addition of standard or samples plus monoclonal to plate.** Samples or standards plus monoclonal antibody (200 μ L) were added to the plate, which was then incubated for 90 minutes at 37°C in the dark. The wells were aspirated and washed four times with PBS/Tween. **Addition of goat anti-mouse antibody-biotin conjugate.** Diluted antibody-biotin conjugate (200 μ L) were added to each well, and the plate was incubated for 90 minutes at 37°C in the dark. The wells were aspirated and washed four times with PBS/Tween. **Addition of Streptavidin-Poly-HRP conjugate.** Diluted streptavidin-Poly-HRP conjugate (200 μ L) was added to each well, and the plate was incubated for 90 minutes at 37°C in the dark. The wells were aspirated, washed four times with PBS/Tween, and once with 300 μ L/well of 100 mM $\text{Na}_2\text{H}_2\text{O}_2$ to remove potassium salt. **TMB reaction.** TMB solution (200 μ L) was added to each well, and the blue color was allowed to develop at room temperature in the dark until a B₀ absorbance of approximately 0.40 at 630 nm was obtained. Forty microliters of a 1.5 N H_2SO_4 solution were added to each well to stop the reaction, and the absorbance was read at 450 nm. A logit transformation was performed on the absorbencies of the standards to linearize the calibration curve. The

quantity of (+)-ABA in the samples was calculated by performing the logit transformation and comparing the results to the calibration curve of (+)-ABA for each plate.

GC/MS-SIM Validation of the indirect ELISA. Samples extracted for validation of the indirect ELISA were purified as stated above and taken to complete dryness using rotoevaporation in vacuo at 37°C and dim lighting. Samples were methylated with ethereal diazomethane, and ABA levels were quantified by GC/MS-SIM using $^2\text{H}_6$ -ABA as an internal standard. The analysis was performed on a Hewlett-Packard 5890 GC with capillary direct interface to a Hewlett-Packard 5971A mass-selective detector. The GC program was 60°C for 2 min., 25°C/min. to 165°C, then 5°C/min. to 275°C. The helium gas flow was 0.5 mL/min. The capillary column was a DB1 (J&W Scientific, Ltd.) 15 m x 0.25 mm x 0.25 μm film. The injection and interface temperatures were 300°C. Data were collected using the SIM program, monitoring four ions: m/e 190 (endogenous ABA), m/e 194 ($^2\text{H}_6$ -ABA), and m/e 162 and 166 to monitor impurities. Retention time for $^2\text{H}_6$ -ABA was 12.34 min. and for ABA was 12.37 min. A calibration curve for $^2\text{H}_6$ -ABA was prepared by monitoring the 190/194 ratios for 308 ng $^2\text{H}_6$ -ABA in standard samples of ABA of 50, 100, 200, 400, 600, 800, and 1000 ng. The amount of ABA in the samples was estimated by entering the 190/194 ratio peak areas into the regression equation of the calibration curve.

Statistical Analysis. Statistical significance of the multiple pairwise comparisons for ABA in the tissues was based on Bonferroni-Welch which assumes normal distributions but unequal variances between samples. Analysis was carried out at 95% confidence intervals ($\alpha=0.05$).

RESULTS AND DISCUSSION

ABA Levels in Developing Seeds

ABA content is usually expressed on a per tissue or on a concentration basis (i.e., dry or fresh weight). Both techniques have advantages and limitations. In this study, concentration on a dry weight basis was chosen due to the necessity of freezing and storing the tissues as quickly as possible. "Per tissue" basis results were determined by dividing total dry weight by the number of embryos or megagametophytes collected (i.e., "average" embryo or megagametophyte basis).

Indirect ELISA was selected as the method of analysis because of its high specificity and sensitivity toward ABA (1 pg/100 μL), and due to the small amounts of embryo tissue (< 0.3 mg dry weight) that were available in the early stages of development (Stages 3-7). ELISA validation by GC/MS-SIM using late stage tissue demonstrated good agreement between the two methods, and showed no crossreactants or interferences to the ELISA (data not presented).

Changes in ABA levels found in developing embryos and megagametophytes are illustrated for "UC" in Figures 1 and 2 on a per tissue and dry weight basis, respectively. ABA was detected in all tissues tested. In many herbaceous species, ABA has been found to be extremely low or undetectable during early development, increasing to a maximum level at one-third to one-half maturation with subsequent decreases back to low levels at maturity (Black 1991). This trend was not found on a per tissue basis in loblolly pine. There were no significant changes in ABA found in megagametophytes during development, except for tissue at Stages 9A and 9B which were significantly higher. In embryos, there was a significant increase in ABA from Stages 8 to 9A, followed by a decline and a second increase at 9F. The first increase was simultaneous with the apparent increase in the megagametophyte, inferring ABA in embryos was imported from the megagametophyte. In other species, it is not known whether the measured ABA was synthesized by

seed tissues or imported from the mother plant (Brenner 1986). ABA in the megagametophyte was significantly higher than in the corresponding embryo tissue, a trend that appears to be species-dependent. In *Zea mays* L., ABA in the embryo was found to be higher than in the endosperm (Jones and Brenner 1987), while the opposite was found in (*Prunus persica* L.) (Piaggese et al. 1991).

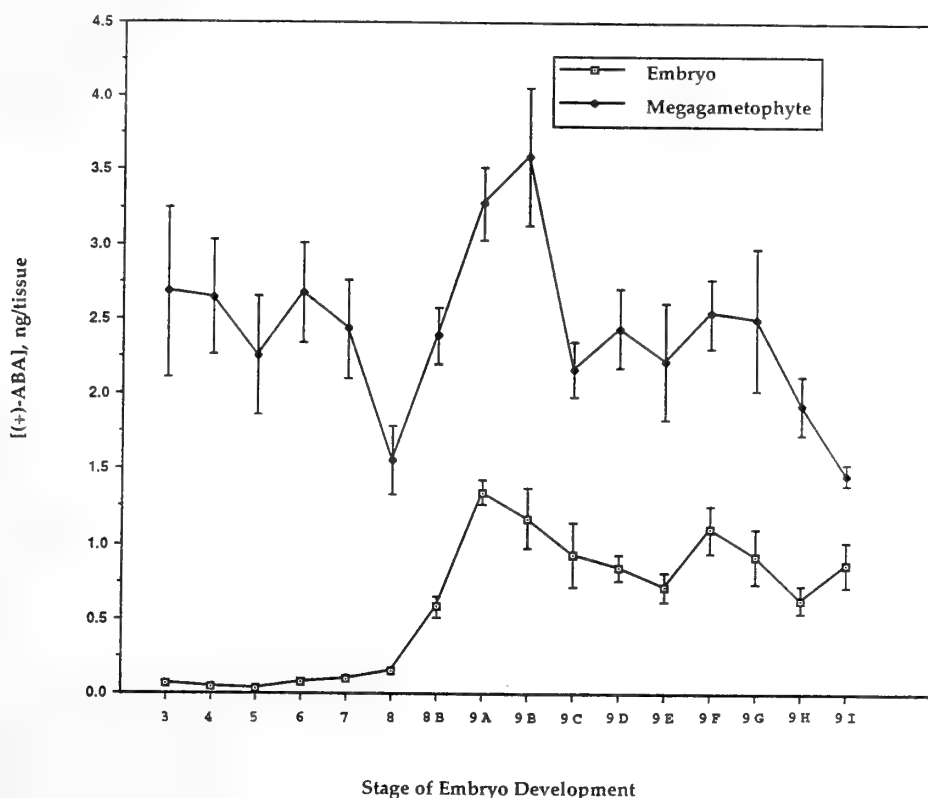


Figure 1: ABA levels in developing embryos and megagametophyte from the "UC" from stage 3 to cone ripening (June-October 1992), on a per "tissue" basis. Stage 9 tissue was divided into additional substages according to percent moisture content of embryo/megagametophyte, respectively: A: 62/42%, B: 57/38%, C: 44/31%, D: 35/30%, E: 34/28%, F: 30/25%, G: 32/24%, H: 31/25%, I: 30/25%. Error bars represent 95% confidence limits.

The generalization reported by Black (1991) does not apply for ABA per dry weight as well (Figure 2). Overall, the highest levels of ABA in embryos were found at early development with a steady decline to cone ripening. There appears to be at least two significant peaks (Stages 7 and 9F), possibly four (Stages 4 and 9A) for ABA in embryos of loblolly pine. Multiple peaks for ABA are not uncommon, and it has been reported that endogenous ABA levels can exhibit two peaks in embryos of developing *Phaseolus coccineus* (Hsu 1979) and *P. vulgaris* L. (Perata et al. 1990) and three to four peaks in *Brassica napus* L. (Finkelstein et al. 1985). However, these peaks do not necessarily occur at the same point during seed development.

In the work to date with "UC", the ABA peaks do not appear to correspond to any major morphogenic changes in the embryos, e.g., development of cotyledons. The ABA determined in embryos is significantly higher than that in the megagametophyte tissue. Again, this trend appears to be species-dependent. Although similar trends have been demonstrated with *Hordeum vulgare* L. (Quarrie et al 1988) and *Zea mays* L. (Jones and Brenner 1987), the opposite was found in *Medicago sativa* L. (Xu et al. 1990). It would appear that ABA in

loblolly pine megagametophytes does not change (Figure 1), and the decline in ABA depicted in Figure 2 is caused by an increase in dry weight during development rather than by a drop in ABA.

ABA Levels and Dry Weight Accumulation in Developing Embryos

Changes in embryo ABA relative to dry weight displayed in Figure 3. A rapid increase in ABA occurred just prior to that in dry weight. This trend has been observed in many species, including *Triticum*, *Bassica*, and *Glycine* (Black 1991) and *Zea* (Jones and Brenner 1987). Although a cause-and-effect relationship is suggested, the increase in ABA levels cannot be said to cause the increase in loblolly pine embryo dry mass without further evidence.

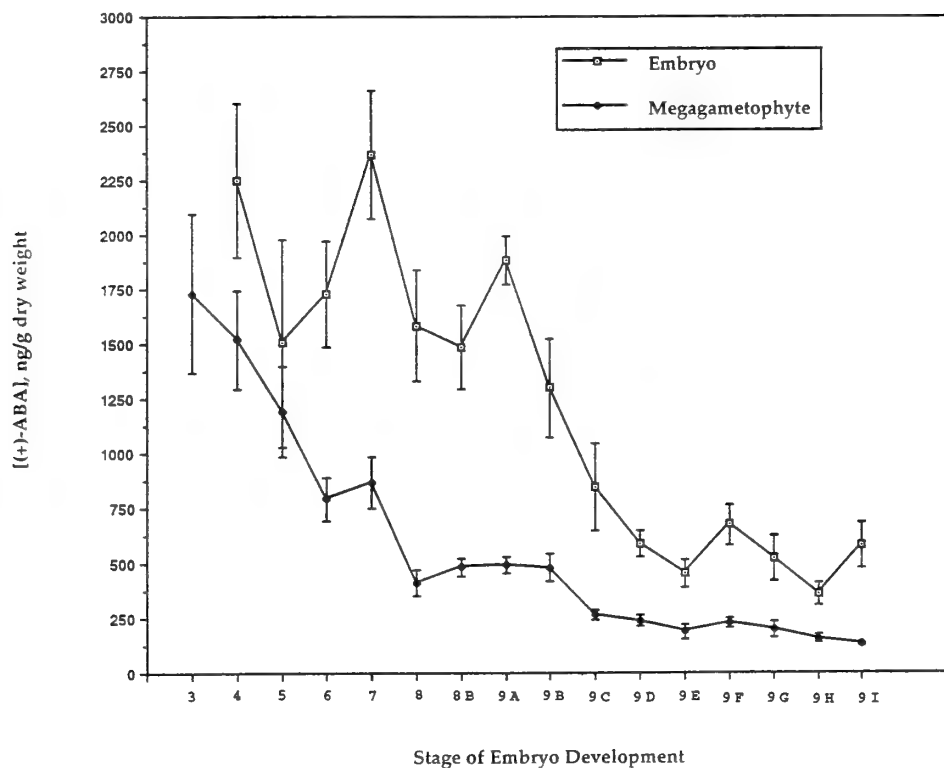


Figure 2. ABA levels in developing embryos and megagametophyte from the "UC" from Stage 3 to cone ripening (June-October 1992), on a per g dry weight basis. Stage 9 tissue was divided into additional substages according to percent moisture content. See Figure 1 for description of substages. Error bars represent 95% confidence limits.

SUMMARY AND CONCLUSION

Results for the single clone analyzed to date suggest that ABA in megagametophytes (on a tissue basis) remains constant during seed development and maturation. ABA in embryos remained low during early to middle stages of development, increased to its highest level at Stage 9A, then steadily declined and peaked a second time at 9F. Changes in embryo ABA levels did not appear related to any major morphogenic event, but the major increase observed midway through development preceded exponential growth and dry weight accumulation.

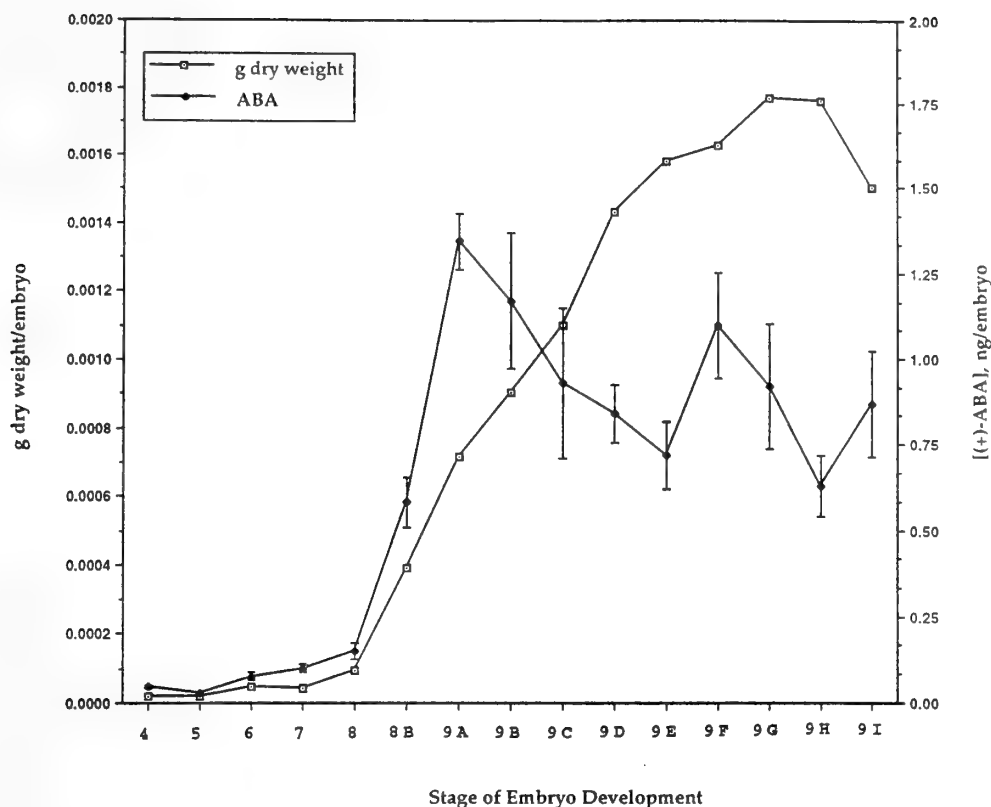


Figure 3. ABA level versus dry weight accumulation in developing embryos from the "UC" from stage 3 to cone ripening (June-October 1992). Stage 9 tissue was divided into additional substages according to percent moisture content. See Figure 1 for description of substages. Error bars represent 95% confidence limits.

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EMBRYO EXPLANT INFLUENCE ON CALLUS INITIATION
FREQUENCY FOR LOBLOLLY PINE

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Abstract. --The objective of this study was to compare callus initiation frequency in response to different culture methods of loblolly pine (*Pinus taeda*) embryo explants. Cones were collected at the end of August and September, 1992, from trees in Arkansas. The seeds were isolated, disinfected in 70% ethanol for 30 s and in 30% Clorox for 15 min, and rinsed in sterilized water. Seed coat was removed, and the embryos were inoculated either attached to or separated from the megagametophytes. The separated megagametophytes were also cultured to test their potential to produce callus. These explants were cultured on half-strength modified MS medium adjusted to pH 5.8 and supplemented with 11 mg/L 2,4-D, 4.5 mg/L kinetin, and 4.5 mg/L BAP. The cultures were maintained in the dark at 20±3°C for 4 weeks, after which callus formation was recorded. Callus initiation frequencies from seeds collected in August and in September, respectively, were 78.3% and 92.5% for embryos, 13.7% and 27.2% for megagametophytes, and 29.9% and 48.6% for embryos attached to the megagametophyte. These differences indicate that callus initiation frequency from isolated embryos was higher than the frequency obtained from the other explants. These results suggest that the presence of megagametophyte tissue may restrict callus initiation from the embryo.

Keywords: *Pinus taeda* L., megagametophytes, somatic polyembryogenesis, embryogenic callus.

INTRODUCTION

Loblolly pine is an economically important coniferous species in the southeastern United States. It is the main species on about 29 million acres of plantations (Brender et al. 1981). It accounts for half of the nation's volume of southern yellow pine and supports a huge and growing forest products industry.

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Breeding and biotechnology are being integrated into genetic improvement program. Such integration may be more important in programs for woody than for herbaceous species because of the time required for sexual maturity in most woody species (Riemenschneider et al. 1988). Use of asexual propagation could yield gains more than twice as fast as those from sexual propagation, with gains of 18 to 32% in volume per acre for first generation seed orchards alone (Talbert 1982, McKeand et al. 1984). The principal advantage conferred by micropropagation is that plants can be selected for superior performance that results from both additive and non-additive genetic effects. Conventional seeding-oriented methods can effectively utilize only additive genetic gain (Ammirato 1986, Van Buijtenen and Lowes 1989).

Many ongoing loblolly pine improvement programs would benefit from capturing of genetic gain through somatic polyembryogenesis (SPE) of loblolly pine. Somatic polyembryogenesis is a multiplication process in which cells of an embryonal suspensory mass (ESM) differentiate into new embryos according to true-to-type development without excision of cells or tissues from the differentiated body or from the post-proembryonal stages of development. The multiple embryos that normally degenerate during seed development are rescued as an ESM and regenerated new plantlets (Durzan 1988a and 1988b). Compared with organogenesis, SPE produced large numbers of somatic embryos. Economies are expected not only for mass and rapid propagation of elite trees but also for eventual automated, large scale in vitro production (Gupta et al. 1993).

To realize these enormous advantages of SPE in loblolly pine tissue culture, the process must be well defined. Only a few groups have reported some progress on SPE of loblolly pine (Becwar and Feirer 1989, Becwar et al. 1988, 1990, 1991, Gupta and Durzan 1987a and b). The repetitive polyembryogenesis cycle can be induced; however, conversion of the immature somatic embryos to the mature somatic embryo and the recovery of plantlets remains difficult (Beckwar and Feirer 1989). Beckwar et al. (1990) stated that the explant developmental stages are the most important factor for the initiation of embryogenic cultures and somatic embryo development in loblolly pine. Al-Khayri et al. (1992) reported that the optimum periods to collect loblolly pine in our areas are June, July, and August. However, there has been no report on the effects of culture methods of immature loblolly pine embryos on callus induction.

The objectives of this study was to compare different explants from immature seeds of loblolly pine relative to their frequency of callus initiation.

MATERIALS AND METHODS

Cone Collection

Immature cones of loblolly pine were collected from six trees grown at the Arkansas Agriculture Experimental Station, Fayetteville, AR and at Hope, AR. Two collections were made at the end of August and of September during 1992. Four cones were collected from each tree, providing 24 cones at each collection.

Seed Extraction, Disinfection, and Embryo Removal

Following each collection date, scales were removed by peeling away from the axis of the cone with a knife. After being exposed the seeds were removed, placed in a plastic bag, and kept in a refrigerator for several days until all the seeds from the samples had been extracted. The seeds were surface disinfected in 70% ethanol for 30 seconds, emersed for 15 min in 30% Clorox solution containing 0.01% Tween 20, and rinsed 4 times in sterile water. With a scalpel, seed coats were removed exposing the immature embryos.

Explant Tissue Selection

Three types of explant were cultured depending on their isolation methods. They were: 1) The entire immature seeds including the embryo and megagametophyte (EG), 2) the embryo alone (E), and 3) the separated megagametophyte alone (G).

Callus Induction and Initiation Frequency

The explants were all cultured individually in culture tubes (5x100mm) containing 5 ml of half-strength modified MS medium (275mg/L, NH_4NO_3 , 233/mg/L KNO_3 , 220 mg/L $\text{CaCl}_2 \cdot 2 \text{H}_2\text{O}$, 185 mg/L mg $\text{SO}_4 \cdot 7\text{H}_2\text{O}$, 85 mg/L KH_2PO_4 , 18.65 mg/L Na_2EDTA , 13.90 mg/L $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 11.15 mg/L $\text{Mn so}_4 \cdot 4\text{H}_2\text{O}$, 4.3 mg/L $\text{ZnSO}_4 \cdot 4\text{H}_2\text{O}$, 3.1 mg/L H_3Bo_4 , 0.415 mg/L KI , 0.125 mg/L $\text{Na MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.0125 mg/L $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.0125 mg/L $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 1,000mg/L myo-inositol, 0.25mg/L nicotinic acid, 0.25 mg/L Pyridoxine Hcl, 1.0 mg/L thiamine. Hcl, 1.0 mg/L glycine, 450 mg/L L-glutamine, 500 mg/L Casein hydrolysate) adjusted to pH 5.8 and supplemented with 11 mg/L 2, 4-D, 4.5 mg/L kinetin, and 4.5 mg/L BAP. The cultures were maintained by transfer to fresh mediums at 4-week intervals in the dark at $20 \pm 3^\circ\text{C}$ and the callus initiation frequency was recorded.

Cell suspension establishment and maintenance were performed according to Beckwar et al. (1988). Redifferentiation of callus and cytochemical staining procedures were used after Gupta and Durzan (1987a).

RESULTS AND DISCUSSION

Callus initiation frequency in response to the explants culturing method was evident. Cultured embryos alone produced the highest number of calli. The cultured embryo with the megagametophytes included was second high, and the cultured megagametophytes alone was the lowest (Table 1).

Table 1. Frequency of callus initiated from explants of 6 loblolly pine.

Tree	E	G	EG
F1	66.7	20.0	26.3
F2	76.7	22.0	44.4
F8	81.8	14.9	50.0
H2	91.0	28.9	33.3
H4	97.7	22.7	63.0
H15	97.9	25.5	20.0

E-embryo

G-Megagametophytes

F=Fayetteville, AR.

H=Hope, AR.

The frequency of callus initiation was different among six trees and between two locations (Table 1).

Frequency of callus initiation differ between collection made in August and September for the E and G explants (Figure 1).

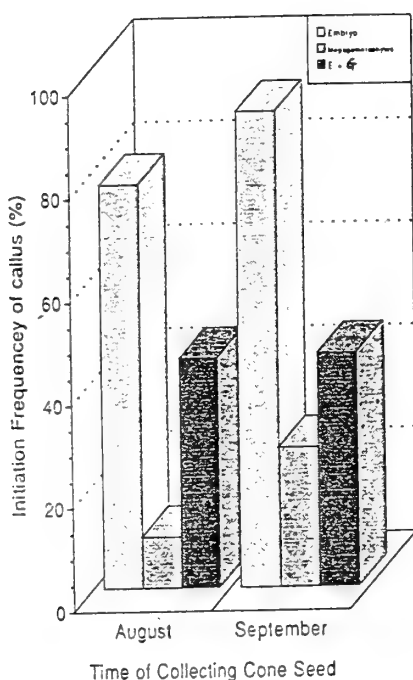


Figure 1. Initiation frequency of callus compared with the time of cone collection and three culture methods.

CONCLUSIONS

These differences indicated that callus initiation frequency from isolated embryos was higher than the frequency obtained from the other explants. This suggested that the presence of megagametophyte tissue may restrict callus initiation from the embryo.

Although the percentage callus initiation showed an indication of the ability of the immature embryos to produce callus, the relationship among cone collecting period, the ability to produce callus, and the capacity to regenerate plantlets have not been established. Further research is needed to identify these relationships.

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FACTORS REGULATING ASEXUAL EMBRYOGENESIS IN VITRO IN LONGLEAF PINE

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Abstract.--Embryogenic cultures of longleaf pine (*Pinus palustris* Mill.) were established from female gametophytes with intact zygotic embryos (at developmental stages 1 and 2) cultured on MSG and DCR basal media in darkness. Induction of embryogenic tissue was observed from explants cultured on both basal media containing glucose, or maltose or sucrose as carbon source. All four levels of each carbon source (15-90 g/L) used supported production of embryogenic tissue. Both auxin (2,4-dichlorophenoxy acetic acid(2,4-D) and cytokinin (N⁶-benzyladenine (BA) at 2 and 1 mg/L; 3 and 0.5 mg/L; 5 and 2.5 mg/L and 2,4-D alone at 10mg/L were effective in producing embryogenic tissue.

Maintenance and proliferation of embryogenic tissue was achieved by transferring extruded embryogenic tissue to half-strength modified MSG medium supplemented with casein hydrolysate (1g/L). The level of 2,4-D was reduced to 0.5 mg/L and BA was eliminated from the medium. This transfer resulted in active proliferation of embryogenic tissue, and cultures were scaled up to 700 tissue masses each weighing approximately 250 mg. These embryogenic tissue masses have been subcultured 23 times to date for a period of 8 months.

Embryogenic tissue examined microscopically both at 3 or 4 weeks and 8 months after initiation revealed somatic embryos at several early stages of development. Somatic embryos from tissues on initiation medium were morphologically different from those on maintenance or proliferation medium. All somatic embryos were pre-cotyledonary and resembled their zygotic embryo counterparts at early stages of development.

Keywords: *Pinus palustris* (Mill.), longleaf pine, asexual embryogenesis, somatic embryogenesis, carbon source, glucose, maltose.

INTRODUCTION

Longleaf pine (*Pinus palustris* Mill.) is an extremely important softwood species in the southeastern United States, showing excellent form, good wood qualities, and resistance to fusiform rust. Substantial genetic variation in traits affecting survival, growth and disease resistance (Schmidtling and White 1989), is seen in longleaf pine, which makes it an excellent candidate for artificial, clonal regeneration. Large scale micropropagation of desirable genotypes of longleaf pine via induction of asexual embryogenesis *in vitro* could offer not only selection of desirable genotypes but also efficient regeneration of genetically transformed plants.

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Additionally, induction of somatic embryos at various developmental stages could become useful for mass production of thin layers of tissue for gene transfer via microprojectile bombardment technique (Sanford 1988).

Reports of somatic embryogenesis in conifers in general, and family Pinaceae in particular are mostly confined to the genera Pinus and Picea, although Larix, Pseudotsuga (Nagmani and Bonga 1985; Nagmani and Dinus 1991 and Nagmani et al. 1991), and Abies (Norgaard et al. 1992) can be reproduced via somatic embryogenesis. Tautorius et al. (1991) and Atree and Fowke (1991) have updated reports of somatic embryogenesis in conifers.

In this paper, we report the factors regulating initiation, maintenance, and multiplication of somatic embryos in longleaf pine and morphological variations of somatic embryos observed in cultures.

MATERIALS AND METHODS

Explants for initiation of embryogenic cultures were obtained from seed cones of longleaf pine collected on July 14 and 27, 1992 from open-pollinated tree growing in the Harrison Experimental Forest, Gulfport, MS. Aseptic techniques used for excision of explants and their culture were as described earlier for loblolly pine (Becwar et al. 1990) and Douglas-fir (Nagmani et al. 1991).

Female gametophytes containing zygotic embryos at stages 1 or 2 and isolated zygotic embryos primarily at stage 3 of development were cultured on modified Murashige and Skoog basal medium (1962) with glutamine (MSG medium) and DCR medium (Gupta and Durzan 1985).

In addition to the customary use of sucrose as the carbon source, maltose and glucose were also used individually as carbon sources for initiation of embryogenic cultures. These 3 carbon sources were used at 4 levels each (15-90 g/L). Growth regulators used were 2,4-dichlorophenoxy acetic acid (2,4-D) and N^6 -benzyladenine (BA) at 0-10 mg/L both individually and in combination. Casein hydrolysate (1g/L) was used as a supplement in some experiments. Methods of culture and culture conditions were as described in detail previously (Nagmani et al. 1993, In press). Culture medium for maintenance and proliferation of embryogenic tissues were modified according to the methods described for Douglas-fir (Nagmani and Dinus 1991), to sustain growth of the embryogenic tissue.

For microscopic examination, embryogenic tissue was stained with 0.5% toluidine blue in glycerol and photographed under phase contrast microscope. Initiation and development of somatic embryos were followed and compared to zygotic embryos according to the system of zygotic embryo classification proposed by Owens and Molder (1984) and Buchholz and Steimert (1945).

Statistical analysis of the data comparing media effects on initiation frequency of embryogenic tissue was performed using the chi-square test (X^2) (Snedecor and Cochran 1967).

RESULTS AND DISCUSSION

Explant type, developmental stage and time of excision:

"Explant type" here refers to either the female gametophyte containing intact zygotic embryos or isolated zygotic embryos since both these explants were used. Developmental stage of the explant corresponds to the apparent morphological equivalent of the zygotic embryo both within the female gametophyte and in the isolated state. The "time of excision" refers to the time of the year during which the seed cones were collected from longleaf pine tree from which explants were extracted. Embryogenic

response was observed from female gametophytes containing zygotic embryos (33 cultures from a total of 944 explants cultured). None of the isolated zygotic embryos produced embryogenic tissue. Extrusion of the embryogenic tissue was observed from the micropylar region of the female gametophytes in all 33 responsive explants. Somatic embryos were observed in embryogenic tissue masses by 4 weeks in culture (Figure 1A). The developmental stage of the zygotic embryo within the female gametophyte was classified under stages G and H of (Owens and Molder 1984) lodgepole pine embryo classification. We have modified the classification to include stages G and H under stages 1 & 2. The isolated embryos cultured were at stage 3 of development according to the classification proposed by Buccholz and Stiemert (1945) for *Pinus ponderosa*. The time at which zygotic embryos reach certain stage of development is associated with the time of the year during which seed cones are collected for culture. Accordingly, in loblolly pine, explants excised from seed cones collected on July 14, 1992 proved to be generally responsive to initiation of embryogenic tissue. Isolated embryos cultured were at stage 3 of development, and were excised from seed cones collected on July 27. As the explant type, age and time of excision are interrelated, it is difficult to ascertain the role of each one of these three factors individually on initiation frequency of embryogenic cultures. However, loblolly pine female gametophytes containing embryos at stage 1 or 2 of development were responsive, in contrast to isolated embryos as explants. This may be partly due to nutritional support provided by gametophytic tissue. Reports of successful initiation of embryogenic cultures in other pine species such as *P. strobus* (Finer et al 1989), *P. taeda* (Becwar et al. 1990) *P. radiata* (Smith 1987) and *P. elliottii* (Jain et al. 1989) suggest that pre-cotyledonary zygotic embryos were most responsive to initiation of embryogenic tissue. However in *Picea*, cotyledonary embryos initiated embryogenic cultures (Hakman et al. 1985).

Effect of carbohydrates

Maltose, glucose and sucrose were equally effective in the induction of embryogenic tissue from the explants. Out of a total of 248 explants cultured on media containing glucose (at 4 levels) 4 explants produced embryogenic tissue. Maltose-containing media (at 4 levels) supported initiation of embryogenic tissue from 18 out of a total of 472 explants cultured. Media with sucrose as the carbon source, induced embryogenic tissue in 11 of 224 explants.

Effect of basal media

Only one of 192 explants cultured on growth regulator free basal media (modified MSG and DCR media) produced embryogenic tissue. There was no significant difference between MSG and DCR basal media used as far as embryogenic response of the explants. The addition of casein hydrolysate to the medium was not necessary for initiation of embryogenic tissue.

Effect of growth regulators

Combinations of 2,4-D and BA at 2 and 1 mg/L, 3 and 0.5 mg/L, 5 and 2.5 mg/L respectively were effective in the induction of embryogenic tissue. When 2,4-D was used alone at 5 and 10 mg/L, 4 explants out of 96 cultured produced embryogenic tissue at 10 mg/L of 2,4-D, while no response was seen at 5 mg/L.

Maintenance and proliferation

For maintenance and proliferation of embryogenic tissue, the concentrations of macro- and micro-nutrients in MSG medium were reduced to half-strength and casein hydrolysate (1g) was added as supplement. Further, the level of 2,4-D was reduced to 0.5 mg and BA was deleted. This resulted in proliferation of embryogenic tissue, and the cultures were scaled up to about 700 tissue masses each weighing approximately 250 mg.

Morphological variations of asexual embryogeny in vitro

Histological examination of embryogenic tissue at both 7-weeks and 8 months after initiation revealed somatic embryos at various stages of development (Figures 1B-I). Fig 1B-D shows somatic embryos from 7-week

-old embryogenic tissue. Figure 1B shows two somatic embryos with long, coiled suspensors and an embryonal mass (em) with 4, 12 or 24 cells which are densely cytoplasmic with prominent centrally located nuclei. These cells are compact and well defined. The cells of the suspensor (sc) are considerably enlarged and vacuolated with sparse peripheral cytoplasm (Figure 1C). The cells of the embryonal mass and suspensor are shown to have divided further resulting in multicellular embryonal head (eh) and multilayered suspensor (Figure 1D).

Figures 1E-I show somatic embryos from 8-month-old embryogenic cultures on maintenance and proliferation media. The embryogenic tissue masses were subcultured 23 times at 10-15 day intervals for a period of 8-months onto half-strength modified MSG medium with 2,4-D at 0.5 mg/L.

Figures 1E,F represent 2 stages of early somatic embryo development where an embryonal mass (em) of 2, 4 and 6 cells with prominent nuclei and dense cytoplasm are subtended by 1 or 2 tiers of broad suspensor cells (sc). Figures 1G-I show morphological variations of early somatic embryos with loosely arranged cells in the embryonal mass (em) and elongated suspensor cells (sc). Early somatic embryos shown in Figures 1E,H resemble their zygotic embryo counterparts documented and illustrated for a conifer species (Konar and Nagmani 1980). Also, early somatic embryos documented in Figures 1B,C resemble in early somatic embryos of Norway spruce (Nagmani et al. 1987). Some embryogenic tissue masses of longleaf pine have been transferred to development and maturation media to further development of somatic embryos.

(Figures 1A-I here)

Early somatic embryogeny in longleaf pine differs from early zygotic embryogeny in pine species in that the former lacks the free nuclear division common to post-fertilization events in the zygote. In somatic embryogeny the early somatic embryos are cellular bipolar structures with embryonal mass subtended by suspensor cells.

Morphological variations are seen between somatic embryos on initiation, maintenance and proliferation media. Somatic embryos at pre-cotyledonary stages from embryogenic cultures on initiation media have long coiled massive suspensors at early stages of development (Figure 1B) as compared to somatic embryos from cultures on maintenance and proliferation media (Figures 1E-I). We speculate that early stages of embryo development observed in cultures on maintenance and proliferation media occur due to either continual de novo initiation from callus cells on maintenance and proliferation media or proliferation from pre-existing asexual embryos. Somatic embryos observed on initiation media (Figures 1B-D) represent rather advanced stages of early embryo development as compared to cultures on maintenance media (Figures 1E-I). This may be due to the relative age of embryogenic cultures where 7-week-old embryogenic tissue on initiation medium is exposed to auxin (2,4-D) in the medium for a shorter period of time (7-weeks) as compared to 8-month-old embryogenic tissue on maintenance medium (subcultured 23 times for a period of 8 months) exposed to auxin (2,4-D) for longer period of time. Transfer of these embryogenic cultures to a medium either free of auxin or to a medium with no auxin but with abscissic acid (ABA) might promote development of somatic embryos to cotyledonary stages of development. Experiments are underway to test this hypothesis.

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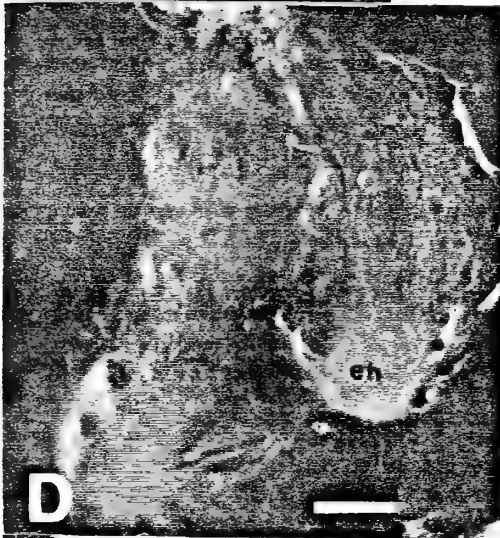
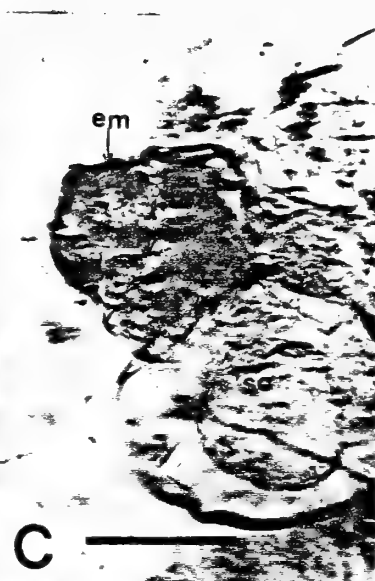
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FIGURE CAPTIONS

Figure 1. Somatic embryos of longleaf pine.

- A. 7-week-old embryogenic tissue on induction medium showing somatic embryo (se). Scale bar= 1mm.
- B. Stage 1 somatic embryos showing an embryonal mass (em) and long coiled suspensor system (ss) on induction medium. Scale bar= 10 μ m.
- C. Somatic embryo enlarged showing compact embryonal mass (em) and broad suspensor cells (sc). Scale bar= 0.1mm.
- D. Stage 2 somatic embryos with an embryonal head (eh) and multilayered suspensor system (ss). Scale bar= 0.5mm.
- E. 4 or 8-celled somatic proembryo with 2-4 embryonal cells (ec) subtended by 2-4 suspensor cells (sc). Scale bar= 0.1mm.
- F. Somatic proembryo with 2 tiers of suspensor cells (st) and an embryonal mass (em). Scale bar= 0.1mm.
- G,H. Morphological variations of 4 or 8 celled somatic proembryo with an embryonal mass (em) subtended by 2 or 4 broad or elongated suspensor cells (sc). Scale bar= 0.1mm.
- I. Variation of stage 1 somatic embryo with an embryonal mass of loosely arranged cells (em) and long suspensor system (ss). Scale bar= 0.1mm.



STEM-CUTTING PRODUCTION AND ROOTING PERFORMANCE IN AN S2 POPULATION OF LOBLOLLY PINE¹

C.D. Nelson, T.D. Caldwell, and J.M. Hamaker²

Abstract.-- An inbred population of loblolly pine (*Pinus taeda* L.) was formed by self-pollinating for two generations. The first generation (S1) was grown in field plots on the Harrison Experimental Forest (HEF) in south Mississippi. All flowering (female and male) S1 trees, 16 years after planting, were self-pollinated to produce the second generation (S2). Seedlings of the S2 families and a related outcross control family were repeatedly hedged to promote the production of multiple shoots. On each of nine hedging dates, the shoots were collected and set for rooting under intermittent mist. Each seedling hedge was scored for the number of useable cuttings produced and for the number of cuttings that later developed supporting root systems. The average number of useable cuttings per seedling increased from 1.0 to 22.2, while the proportion of cuttings that rooted decreased from 0.41 (in cycle 2) to 0.01. All families responded similarly over the nine cycles for both traits. The differences between the outcross control and the S2 families were significant for cutting production in six cycles and non-significant for proportion rooted in all cycles. The poor rooting response to this rapid hedging and propagation system was unexpected and was not observed in a comparable study of S1 and outcrossed families of slash pine (*P. elliottii* Engelm. var. *elliottii*). Additional studies will be necessary to determine the cause of this response and whether the response applies to a wider range of loblolly pine genotypes.

Keywords: vegetative propagation, inbreeding, *Pinus taeda* L.

INTRODUCTION

Inbred line development followed by hybrid breeding has several drawbacks as an improvement method for the southern pines (Franklin 1969a, 1969b; Snyder 1972; Snieszko and Zobel 1986). Some of these drawbacks are similar to those encountered in corn improvement where the inbreeding-hybridization method is preferred. Both species are highly heterozygous and, as a result, suffer from severe inbreeding depression, making line development and testing difficult. Additionally, both species are extremely difficult to propagate clonally, reducing the opportunity for within-line selection and presenting problems with the commercial increase of promising hybrids. The most obvious drawback to the inbreeding-hybridization method for southern pine improvement is the generation length, which is currently 5 to 10 years under optimal conditions.

Beyond inbred line development for hybrid breeding purposes, inbreeding can produce useful stocks for genetic research. Inbred materials are useful for calibrating genetic diversity measures (Kuhnlein et al. 1990), revealing novel mutants available for genetic mapping or gene cloning (Franklin 1969c), developing near isogenic lines for gene function and transformation studies, and for producing homogeneous seed-propagated populations. Obviously, most of the

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same drawbacks that apply to the inbreeding-hybridization improvement method also apply to inbred line development for genetic research purposes. However, the potential benefits seem to outweigh the drawbacks, especially considering the recent technological advances in molecular genetics, and accelerated breeding and clonal propagation of the southern pines (Nance and Nelson 1989).

In light of these advances, we have initiated an inbreeding program within a loblolly pine population of east Texas origin. The objective of this program is to produce as many multi-generation, self-pollinated lines as possible in as short a time as possible. Tests of inbreeding depression will be made during this process in an effort to develop performance versus homozygosity (inbreeding coefficient) curves for several traits. Toward this end, we are currently producing S0, S1, S2, and S3 seed within the study population. Here we describe results from a vegetative propagation study in which we repeatedly removed all shoots (stem-cuttings) from six S2 families and an outcrossed control family and evaluated the families and clones for stem-cutting production and rooting performance.

MATERIALS AND METHODS

Population

Founding parent trees were originally selected (randomly) as parents in a 10-tree half-diallel mating design involving 6 seed sources (Synder 1967). Trees in two sources— Conroe and Nacogdoches, Texas— were self- and cross-pollinated according to a half-diallel design with selfs. The self-pollinated families (S1) were sown in the Harrison Experimental Forest (HEF) nursery (20 miles north of Gulfport, MS) and outplanted to a field site on the HEF. After 16 years in the field, all flowering S1 trees were self-pollinated (February 1988) to produce the S2 generation (F , inbreeding coefficient, $=0.75$). Fourteen trees were flowering, thirteen with female and male flowers and one with female only. The female-flower-only tree was pollinated with pollen from eight unrelated S1 trees. At cone-collection time, the cones from the different crosses on this tree were mixed, effectively resulting in a pollen-mix (half-sib) family ($F=0$). Table 1 gives the pollination and germination data for the 13 S2 crosses and the 1 outcross.

All S2 families and the outcross control family were germinated after cold stratification and sown in Ray Leach pine (4 in³) cells (April 1990). For families with 22 or less sound seeds (Table 1), all seeds were used. Twenty seeds were used for the other S2 families, and 50 seeds for the outcross control. The seedlings were hedged initially 9 weeks after germination and later transplanted to the HEF Cutting Orchard. Several of these genotypes in each family will be clonally propagated as rooted cuttings and moved into the accelerated breeding program to serve as parents for the S3 generation.

Experiment and Propagation Methods

Remnant seeds from 6 of the 13 S2 families and the outcross control family were stratified, germinated, and sown in Ray Leach stubby (7 in³) cells (June 1991). Following germination, the 6 S2 families were represented by 12 to 29 seedlings and the outcross control by 38 seedlings. These seedlings were arranged into a randomized complete block (RCB) design with four replications, placing one-quarter of the seedlings from each family in each replication. Over a 19-month period (October 1991 to April 1993), the seedlings were hedged 9 times (Table 2). The length of time between hedgings varied depending on the population's rate of response to the hedging. When most (approximately 90% based on visual examination) of the shoots had elongated to at least 7 cm, the seedlings were hedged. In March 1992, after the third hedging, the seedling hedges were transplanted to 3.78-L pots containing pine bark, peat, and vermiculite (2:1:1) and moved outdoors.

Table 1. Identification, seed, and germination data for the 14 S1 loblolly pine parent trees.

S1 family ID ^a	SIFG clone ^b	seed extracted	sound seed ^c	sound seed (%)	germinated / sown
2-5	1238	4500	120	2.7	13/20
2-5	1239	415	22	5.3	16/20
3-7	1240	2685	108	4.0	14/20
3-5	1241	100	13	13.0	9/13
3-4	1242 ^d	350	13	3.7	8/13
2-8b	1243	1290	21	1.6	18/21
2-8b	1244	2500	61	2.4	18/20
3-8	1245	3350	90	2.7	13/20
3-8	1246	1835	96	5.2	18/20
2-5	1247	715	41	5.7	17/20
2-5	1248 ^d	23	2	8.7	2/2
3-7	1249 ^e	835	398	47.7	47/50
2-8a	1250	435	19	4.4	17/19
2-8b	1251	180	10	5.6	8/10
S2 family totals ^d		18378	616	3.35	171/220

^a Source ID - Parent tree (within-source) ID from the original half-diallel mating design (Synder 1967), where source 2 is Conroe, TX, and source 3 is Nacogdoches, TX.

^b SIFG (Southern Institute of Forest Genetics) clone ID number (Mason et al. 1993), clones with the same S1 family ID are full-siblings.

^c Number of seeds remaining after floating in distilled water (estimate of the number of filled seeds).

^d Clone 1242 produced very little pollen and most cones on clone 1248 were damaged by pollen isolation bags.

^e Clone 1249 was cross-pollinated with 8 unrelated S1 pollens and is excluded from the totals.

On each hedging date, the number of useable cuttings (> 7 cm) for stem-cutting propagation were counted (#Cutt) and removed, and a randomly selected subset of these cuttings was immediately set for propagation. The subset included all cuttings for the first 4 hedgings (propagation cycles) and 10 or 15 cuttings per seedling for the last 5 cycles (Table 2). The cuttings were dipped in Hormodin 2 (a commercially available rooting powder containing 0.3% IBA), set in Ray Leach fir (3 in³) cells containing a 2:3 ratio of peat and perlite, and placed in a propagation greenhouse. Moderate temperature ($\leq 85^{\circ}\text{F}$) and high humidity ($\geq 85\%$) were maintained in the greenhouse with evaporative cooling, air conditioning, intermittent mist, and fog. Over the course of the study, both the seedling hedges and their cuttings were maintained in the RCB arrangement. When rooting was nearly complete (Table 2), the cuttings were moved to a standard greenhouse and scored for rooting— number of cuttings with a supporting root system (#Root). From this number, and the number of cuttings set (#Set), the proportion of rooted cuttings (pRoot) was calculated.

Data Analysis

Proportion of cuttings with supporting root systems (pRoot) and number of useable cuttings (#Cutt) were subjected to the analysis of variance procedure using the following models:

Family analysis within cycles--

$$Y = R + F + R \cdot F + E,$$

Family analysis among cycles--

$$Y = P + R(P) + F + P \cdot F + R \cdot F(P) + E,$$

Clone analysis within reps among cycles--

$$Y = P + F + P \cdot F + C(F) + E,$$

where Y is either pRoot or #Cutt, R is replication, F is family, P is propagation cycle, C is clone, and E is error. Analyses were restricted to cycles 2 through 9, since only 1 cutting was available per seedling in cycle 1. In the experimental design, replications were nested in propagation cycles (R(P)), and clones were nested in families (C(F)) and in replications. Thus, the clone analyses were restricted to within replications, resulting in four independent experiments. Prior to analysis, both independent variables were transformed— $\arcsin(\text{pRoot})^{-0.5}$ and $(\text{\#Cutt}+0.5)^{-0.5}$ —to stabilize the error variance (Steel and Torrie 1980). Proc GLM (SAS 1990), with Type III sums of squares, was used to compute the analyses of variance. A weighted analysis was utilized for pRoot, with the number of cuttings set (#Set) used as the weight. Terms involving replication and clone were considered random, while all others were considered fixed. F-tests were computed with the "random" statement and "test" option in proc GLM.

Table 2. Timing and magnitude of the propagation cycles.

Propagation cycle	Date hedged	Elapsed time(days)	Avg. #Set ^a	Avg. #Cutt ^b	Time(wks) in rooting	Avg. pRoot ^c
1	2 Oct 91	65	1.0	1.0	13	0.14
2	15 Nov 91	43	3.9	4.0	14	0.41
3	2 Mar 92	106	3.8	3.8	12	0.12
4	1 May 92	59	8.6	8.6	9	0.03
5	19 Jun 92	49	14.7	16.0	12	0.03
6	30 Jul 92	41	14.0	15.6	8	0.01
7	13 Sep 92	45	9.7	15.9	8	0.01
8	23 Nov 92	66	10.3	15.3	9	0.01
9	13 Apr 93	141	16.1	22.2	11	0.01

^a Average number of cuttings set per seedling per cycle.

^b Average number of useable cuttings per seedling per cycle.

^c Average proportion of cuttings that rooted per cycle.

RESULTS AND DISCUSSION

The percentage of sound seeds among the S2 families was very low, ranging from 1.6 to 8.7 (Table 1). The highest yielding clone, 1238, produced 120 sound seeds out of 4,500 seeds extracted. The outcross seed was 47.7% sound, a value 5.5 to 30.0 times greater than the S2 families. Germination values among the S2 families were moderate to high, ranging from 62% to 100%. The outcross seed attained 94% germination.

The mean number of useable cuttings per seedling hedge for each family is plotted over propagation cycles in Figure 1. The number of useable cuttings increased significantly between cycles 3 and 4, 4 and 5, and 8 and 9. The pattern of increasing number of useable cuttings was consistent for all families. Figure 1 also gives the mean number of useable cuttings per hedge in a comparable study of slash pine. The slash pine seedlings (3 S1 and 11 outcross families) were started 3 months later than the loblolly, but were given essentially the same treatment.

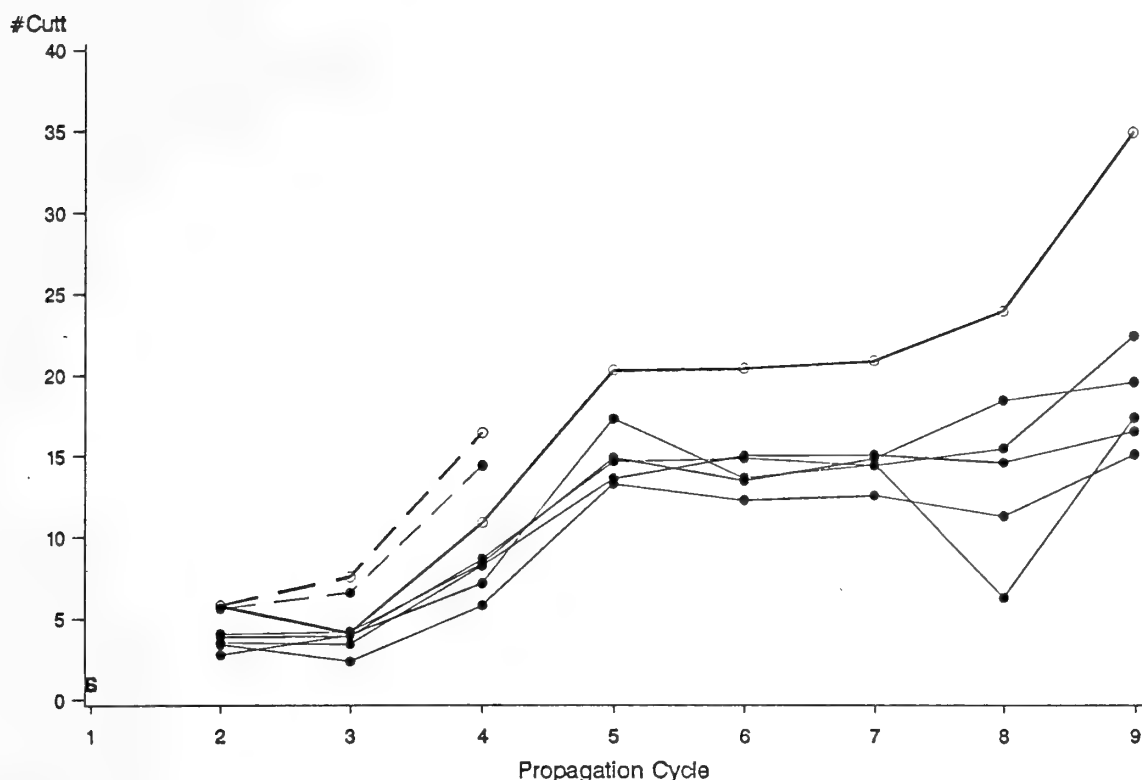


Figure 1. Plot of stem-cutting production (#Cutt) over propagation cycles for 5 S2 and 1 outcrossed family of loblolly pine and S1s (mean of 3 families) and outcrosses (mean of 11 families) of slash pine. Solid lines and 'L' represent loblolly pine and dashed lines and 'S' represent slash pine. Thin lines with filled circles represent the S2 and S1 families and thick lines with empty circles represent the outcross families. The slash pine families were treated similarly and are included as a treatment control.

The mean proportion of cuttings rooted for each family is plotted over propagation cycles in Figure 2. The proportion rooted decreased dramatically between cycles 2 and 3 and 3 and 4. After cycle 4, the rooting proportion remained very low. The same pattern was observed for all families, which was unanticipated, since the rapid method of hedging and propagating was expected to maintain the seedling hedges in a juvenile stage for an extended period of time (5 or more years in a standard hedging system) (T.D. Caldwell, unpublished data).

Figure 2 also gives the mean proportion of cuttings rooted in the slash pine study. The slash pine cuttings received the same treatments and the rooting was completed at the same time in the same propagation greenhouse. However, since the slash pine seedlings were started later, cycles 2 to 5 of the loblolly correspond in time-of-year to cycles 1 to 4 of the slash. The slash pine data for both S1 and outcrossed families showed no decline in proportion rooted through cycle 4 (Figure 2). From both a stem-cutting production and rooting performance perspective, it appears that slash pine will be more responsive to this propagation method.

Results of the family analyses of variance are presented in Table 3. As expected, propagation cycle was the dominant factor. Family was highly significant for both traits; however, the cycle*family and rep*family(cycle) interactions were also significant. Within cycles, family was always non-significant for proportion rooting and nearly always significant for number of useable cuttings (Table 3). The significant family variation was primarily due

to the better performance of the outcross control, especially for number of useable cuttings. The outcross control produced the most cuttings in all cycles and attained the highest rooting proportion in four cycles (Figures 1 and 2). Results of the clone analyses of variance are presented in Table 4. Clone(family) variation was significant only in replication 2 for proportion rooted. In contrast, clone(family) variation was significant in all four replications for number of useable cuttings.

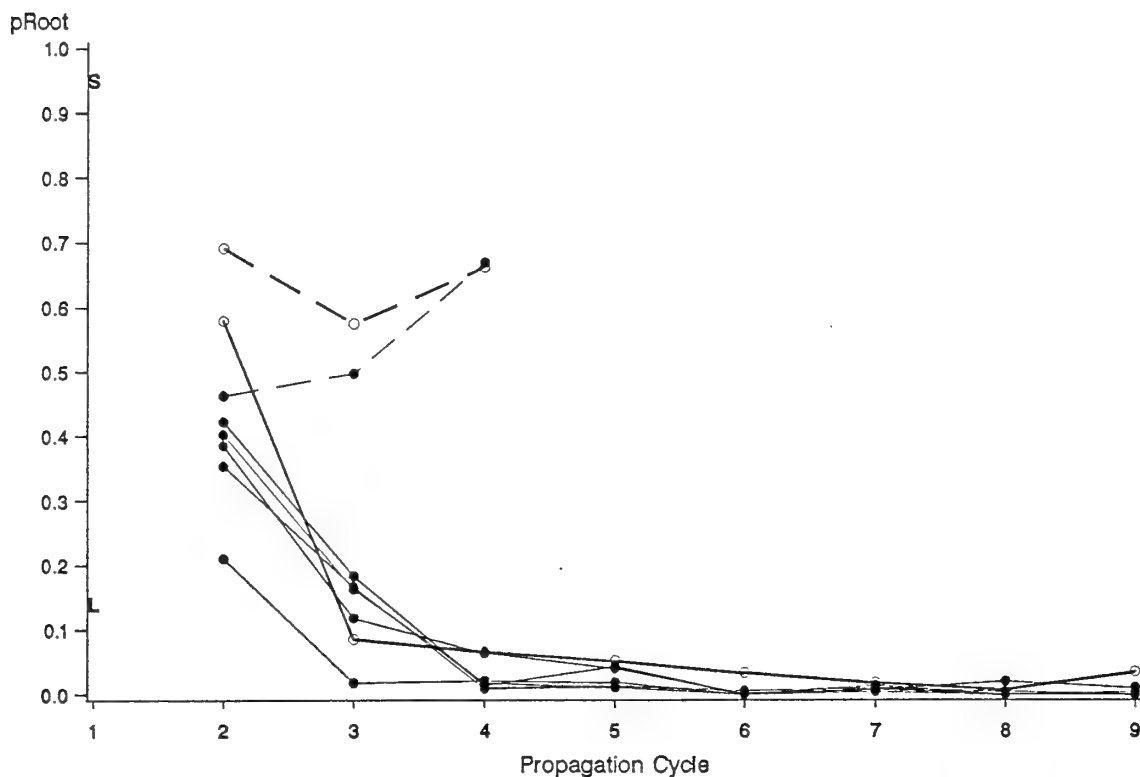


Figure 2. Plot of proportion of cuttings rooted (pRoot) over propagation cycles for 5 S2 and 1 outcrossed family of loblolly pine and S1s (mean of 3 families) and outcrosses (mean of 11 families) of slash pine. Solid lines and 'L' represent loblolly pine and dashed lines and 'S' represent slash pine. Thin lines with filled circles represent the S2 and S1 families and thick lines with empty circles represent the outcross families. The slash pine families were treated similarly and are included as a treatment control.

The results of this study suggest a problem with the application of a rapid hedging and propagating system to loblolly pine. The frequent hedges appear to have detrimentally affected the seedling hedges' potential to produce rootable cuttings. The number of useable cuttings increased, as expected, through cycle 5, then leveled off through cycle 8, and then increased again during cycle 9. The large increase in cycle 9 suggests that the hedges' potential to produce increasing numbers of cuttings was left intact. In the study, cycle 8 preceded and cycle 9 succeeded the first dormant season for the hedges. The increase in number of useable cuttings from cycle 8 to 9 was probably due to the occurrence of favorable physiological changes in the hedges induced by the intervening dormant season. For the same reason, we surmised that rooting performance would recover considerably in cycle 9, but it did not. Thus, the rootability loss does not appear to be closely related to dormancy or season. The seedling hedges will be outplanted to the HEF Cutting Orchard and periodically tested to further investigate these time trends in stem-cutting production and rooting performance.

Table 3. Results of the family analyses of variance of number of useable cuttings (#Cutt) and proportion of cuttings rooted (pRoot).

Family analysis among cycles--					
Source	df	#Cutt MS	F	pRoot MS	F
Cycle	7 ^a	117	98.1***	21.4	55.9***
Rep(Cycle)	24	1.20	1.80*	0.40	1.68*
Family	5 ^b	20.1	29.3***	0.97	4.31***
Cycle*Family	35	1.27	1.85**	0.41	1.66*
Rep*Family(Cycle)	120	0.69	1.48**	0.25	1.52***
Error	962	0.47		0.16	

Notes: Based on the family analysis within cycles model (see Materials and Methods)—Rep*Family was significant ($p=0.05$) in Cycles 3, 4, and 8 and Family was significant ($p=0.05$) in Cycles 2, 4, 5, 6, 7, and 9 for #Cutt; and Rep*Family was significant ($p=0.05$) in Cycles 2, 4, and 6 and Family was non-significant ($p=0.05$) in all Cycles for pRoot. The outcross control was highest in #Cutt in all eight cycles and highest in pRoot in cycles 2, 6, 7, and 9.

* significant at 0.05 level

** significant at 0.01 level

*** significant at 0.001 level

^a Cycles 2 through 9.

^b One S2 family was excluded from the analyses due to low number of seedling hedges.

Table 4. Results of the clone analyses of variance of number of useable cuttings (#Cutt) and proportion of cuttings rooted (pRoot).

Clone analysis within reps among cycles--						
Rep	Source	df	#Cutt MS	F-test	pRoot MS	F-test
1	Clone(Family)	32	1.92	***	0.10	ns
	Error	204	0.28		0.13	
2	Clone(Family)	30	1.57	***	0.30	**
	Error	193	0.28		0.15	
3	Clone(Family)	29	1.40	***	0.17	ns
	Error	195	0.28		0.22	
4	Clone(Family)	40	1.23	***	0.11	ns
	Error	239	0.37		0.15	

Notes: Statistical model included Cycle, Family, and Cycle*Family (see Materials and Methods). F-tests were computed for Clone(Family), only.

ns Non-significant at 0.05 level.

** Significant at 0.01 level.

*** Significant at 0.001 level.

Additional experiments will be required to determine whether the fast decline in rooting performance is applicable only to the studied inbred loblolly pine population or to a wider range of inbred and outcrossed loblolly genotypes. The similar performance pattern of the outcrossed control family to the S2 families suggests that inbreeding depression is not the primary cause of the reduction in rooting performance. However, it will be necessary to test several inbred lines, each represented by inbred and outcrossed seedlings of each generation, to adequately estimate the effect of inbreeding. To accomplish this, we are currently breeding this loblolly pine population to produce S0, S1, S2, and S3 seed. Several of the S2 genotypes cloned as rooted cuttings in the present study will serve as parents for the S3 generation.

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NEEDLELESS SHOOTS AND LOSS OF APICAL DOMINANCE IN GREENHOUSE-GROWN LOBLOLLY PINE

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Abstract.--When loblolly pine (Pinus taeda) is winter planted in the greenhouse and outplanted in May, abnormal growth in the form of multiple apical and needleless shoots has been observed. Results indicate that the tendency to produce abnormal morphology is related to preplanting hardening off. Further, preplanting shortened days and subsequent chilling influenced seedling dormancy and reduced the frequency and severity of abnormal growth.

Keywords: Pinus taeda L., apical dominance, tubelings, abnormal growth

INTRODUCTION

Control crossed loblolly pine (Pinus taeda) tubelings planted in the greenhouse in January and outplanted in May have been observed to produce growth abnormalities after outplanting. Growth abnormalities developed over the course of the summer, and became evident in August. Abnormalities included a loss of apical dominance, the production of needleless shoots, the development of shoots with primary needles, and the production of shoots with sterile scales only (failure of secondary needles to develop). Needleless shoots included both the leader and lateral branches. Symptoms combined to form a needleless basket whorl. Basket whorls often contained a stunted or absent terminal, with long, needleless branches surrounding the terminal. After overwintering, seedlings that expressed the growth abnormalities were observed to resume normal growth. However, several competing leaders were observed on many trees in year two.

Seedlings exhibiting this abnormal growth are of dubious use in the evaluation of progeny tests, and the use of this material could result in erroneous or invalid conclusions about individual tree or family performance. By determining the causes of the observed abnormal growth, procedural changes may be developed to reduce the possibility of invalid conclusions in progeny test evaluation.

Lammas growth, or late season extension of primordia that often produce needleless shoots, was characterized by Rudolph (1964). Hinesley (1982) observed that attempts to bypass the normal chilling requirements of Abies fraseri resulted in stunting, loss of apical dominance, terminal bud abortion, lack of symmetry and other morphological abnormalities. Slee et al. (1976) described needleless shoots, loss of apical dominance, dieback, stem forks, and basket whorls on Pinus caribaea grown in lowland areas of Malaysia. Slee (1977) attributed the presence of the deformities to relatively constant daylength and temperatures near the equator. Out-of-phase dormancy (OPD) influences long shoot growth behavior (Greenwood 1981). Loblolly pine subjected to OPD first cease growth for several months, and later may exhibit needleless shoots, loss of apical dominance, and slow growth.

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Loblolly pine exhibits a mixture of fixed and free growth, producing several flushes during the growing season (Lanner 1976). True dormancy is induced by declining photoperiod and continues until the chilling requirement is satisfied. Boyer and South (1989) found loblolly pine is released from dormancy in early January, following 600 hours of chilling. Garber (1983) found that when buds become dormant before the onset of chilling the chilling requirement may be as little as 400 hours.

To characterize and determine influences on the abnormal growth, two experiments were conducted. The first examined loblolly pine growth response differences from seedling production by two Virginia State agencies. The second experiment examined the effects of preplanting imposed dormancy treatments and the effect of postplanting supplemental water on loblolly pine seedling growth and morphology.

METHODS

Experiment 1: Greenhouse Effects

Fifty seedlings each of five full-sib families (all were Virginia provenances) were raised in either the Virginia Department of Forestry greenhouse in New Kent, Virginia, or the VPI&SU Reynolds Homestead Research Center greenhouse in Critz, Virginia, and later outplanted on the Appomattox-Buckingham State Forest near Appomattox, Virginia.

Germinants were planted into Leach[®] tubes in a mixture of 1:1 peat moss and vermiculite and were fertilized with Osmocot[®] nine-month slow release fertilizer consisting of 18:6:12 N:P:K at a rate of 3 grams of fertilizer per liter of soil. Micronutrients were applied as Micromax[®] micronutrients at a rate of 1.1 grams per liter. Banrot[®] was used to prevent damping off. Lighting was set to mimic daylength over the course of the summer, starting at 13:42 hours, increasing to 14:45 hours on February 19, then decreasing to 13:27 hours by April 23. Seedlings were kept well watered.

New Kent seedlings were placed outside in a seed orchard on April 12, after 14 weeks of growth. Reynolds Homestead seedlings were placed in a lathe house on April 24, after 16 weeks of growth. Seedlings were kept well watered.

On May 18, seedlings were planted on the Appomattox-Buckingham State Forest, on a site that had been clearcut, chopped and burned the previous September. To control competing vegetation, a mixture of Oust[®] (0.224 kg/ha ai) and Roundup[®] (0.456 kg/ha ai) was applied before planting. Seedlings were planted into ten blocks, with factorial combinations of two greenhouses and five families. The experimental unit was the mean value of three seedlings.

Seedlings were examined twice monthly, with root collar diameter (RCD) recorded to the nearest .01 mm, flush height and sterile region height to the nearest mm. The number of sterile scales and the number of needles were counted on each flush. The number and height of lateral branches were recorded. Total final height, the ratio of branch height to total height, and the number of secondary needles per total stem units were calculated. A ratio of branch to apical height of greater than one indicated a loss of dominance. Analysis was conducted using a general linear models procedure (SAS Inst. 1988).

Experiment 2: Imposed Dormancy

Each of six families received factorial combinations of preplanting shortened daylength (0, 4, 6 weeks), and preplanting chilling hours (0, 400, 600 hours), as well as postplanting supplemental water supplied as a split plot. Seedlings were planted in four blocks.

Germinants were planted as before, and grown in the VPI&SU greenhouse facilities in Blacksburg, Virginia. Greenhouse care was administered as described in Experiment 1. On April 4, after 12 weeks of growth, dormancy treatments commenced. Seedlings subjected to 6 weeks of shortened daylength were placed under a heavy (100%) shade cloth for all but 8 hours per day, while the remaining trees were exposed to natural daylength. Seedlings to be subjected to chilling only were placed in a refrigerator at 3°C (with no supplemental light). Seedlings receiving both treatments were first subjected to shortened days and then subjected to chilling. Seedlings were placed in a lathehouse following the conclusion of treatments.

Because of time constraints and to prevent confounding from storage and additional hardening off, seedlings were planted at the Reynolds Homestead Research Center in two shifts. In the first shift (May 25 and 26), all seedlings that were subjected to daylength only, chilling only, and seedlings that did not receive dormancy treatment (controls) were planted. In the second shift (June 11 and 12), all seedlings subjected to daylength and chilling treatments were planted. The site on which the seedlings were planted was an old field, which had been mowed and chemical treated with a mixture of Oust (0.224 kg/ha ai) and Roundup (0.456 kg/ha ai).

Watering commenced on July 8, as it was unnecessary before that. Well watered plots received 1 inch (2.54 cm) of water per week from a soakhose. To reduce herbaceous competition, the site was retreated with Roundup on August 4.

Data were collected and analyzed as described in Experiment 1, above. Data were subjected to analysis of variance, with the two planting dates analyzed separately. As a result of this design, interactions between shortened daylength and chilling treatments could not be completely investigated.

RESULTS AND DISCUSSION

Experiment 1: Greenhouse Effects

Seedlings obtained from the New Kent nursery were initially more stout, yellow in appearance, and were more likely to have a terminal bud than seedlings produced at the Reynolds Homestead. The nursery of origin did not, however, produce differences in height growth (Table 1). Differences in seedling appearance may be attributed to the hardening off period. New Kent seedlings were placed outside two weeks earlier than Reynolds seedlings and were exposed to more direct sunlight in the seed orchard.

Symptoms of abnormal growth were first observed in mid-June, during the development of the second flush. By June 30, approximately 20% of the seedlings measured at Appomattox exhibited a loss of apical dominance, and by the end of the growing season, 85% of the seedlings had lost apical dominance. In most cases, the loss of apical dominance occurred in the most recent flush. Nearly all trees in this experiment completed three flushes, with 56% completing four flushes.

Table 1. Initial height, root collar diameter (RCD), and the frequency of a terminal bud at outplanting for five families of tubelings raised in two greenhouses: the Virginia Department of Forestry greenhouse in New Kent, or the VPI & SU Reynolds Homestead Research Center in Critz. Values in a column followed by the same letter are not significantly different at $\alpha=.05$.

Greenhouse	Height (cm)	RCD (mm)	Bud %
New Kent	23.54 a	3.557 a	80.7 a
Reynolds	23.37 a	3.283 b	40.7 b
Overall Mean	23.45	3.420	60.7

By the end of the first growing season, the greenhouse of origin did not influence height or RCD. However, New Kent seedlings were less likely to lose apical dominance (Table 2). The greenhouse of origin also had an effect on the number of needles per stem unit (Table 2). Again, the New Kent seedlings performed more favorably, expressing more needles per total stem units than the Reynolds seedlings (Table 2). Needle production was influenced by the greenhouse of origin in the second flush, but not in the third. This may be the result of needle formation within the quiescent bud established on the New Kent seedlings before planting.

Differences in greenhouse of origin are interesting because greenhouse treatment and seedling condition were nearly identical. The greatest difference in preplanting treatment of the seedlings was the extended hardening off period for the New Kent seedlings, suggesting that the longer hardening period before planting had a favorable influence on growth.

Table 2. End of growing season growth for tubelings raised in one of two greenhouses (the Virginia Department of Forestry greenhouse in New Kent, or the VPI & SU Reynolds Homestead Research Center greenhouse in Critz) and outplanted on the Appomattox-Buckingham State Forest near Appomattox. Height, root collar diameter (RCD), the number of needles per the total number of stem units for flushes 2 and 3 (N/Unit), and the ratio of branch height to apical height (Final Ratio) are represented. Values within a column followed by the same letter are not significantly different at $\alpha=.05$.

Greenhouse	Height (cm)	RCD (mm)	N/Unit Flush 2	N/Unit Flush 3	Final Ratio
New Kent	40.42 a	12.77 a	0.634 a	0.492 a	1.048 b
Reynolds	39.34 a	13.24 a	0.518 b	0.489 a	1.134 a
Overall Mean	39.88	13.00	0.574	0.491	1.091

Experiment 2: Imposed Dormancy and Supplemental Watering

At the time of outplanting, control seedlings (no dormancy treatments) resembled seedlings raised at Reynolds, as described in Experiment 1. They were slender, generally lacked a bud, and were light green in appearance. This experiment was established at the Reynolds Homestead, where development was generally much slower than at Appomattox. Most seedlings planted at the Reynolds Homestead completed two flushes, with few seedlings completing a third flush. Because growth at the Reynolds Homestead was much slower, expression of growth abnormalities was not as obvious as on the Appomattox site. The summer (especially after planting) was extremely wet; therefore, watering had little effect on the growth of the seedlings.

Shortened days

When outplanted, seedlings subjected to shortened daylengths were smaller and were more likely to have set a terminal bud than the controls. By the end of the growing season, seedlings subjected to four weeks of shortened days did not significantly differ from controls in final height, although they were smaller in diameter. Seedlings subjected to treatment for 6 weeks were both more slender and shorter than control seedlings (Table 3).

Shortened daylength treatments had an influence on needle production in the second flush. Seedlings subjected to shortened days before planting exhibited more needles per total stem units, with the proportion increasing with 4 weeks and again with 6 weeks (Table 3). Shortened days had little influence on the strength of apical control, as reflected in the final ratio of branch to apical height of the seedlings (Table 3).

Table 3. Effect of shortened days before outplanting. End of growing season root collar diameter (RCD), height, needles per total stem units for flush 2 (N/Unit) and the ratio of branch height to apical meristem height (Final ratio). Data represents tubelings exposed to 0, 4, or 6 weeks of 8 hour days before outplanting at the VPI & SU Reynolds Homestead Research Center in Critz. Values within a column followed by the same letter are not significantly different at $\alpha=.05$.

Short Days	RCD	Height	N/Unit Flush 2	Final Ratio
0 Weeks	8.06 a	28.53 a	0.495 c	0.899 a
4 Weeks	7.37 b	27.28 ab	0.680 b	0.809 a
6 Weeks	7.28 b	26.12 b	0.758 a	0.845 a
Overall Mean	7.57	27.31	0.644	0.851

Chilling

Upon outplanting, seedlings subjected to chilling only were shorter than controls and lacked a bud. By the end of the growing season, 400 hours of chilling resulted in a significant reduction of diameter growth, and 600 hours of chilling resulted in a significant reduction of height and diameter growth. Chilling had little effect on needle production, as is indicated by the number of needles per total stem units (Table 4). Although 600 hours significantly reduced seedling growth, the resulting shoots produced a lower branch height to apical height ratio (Table 4), indicating an increase in apical control.

Table 4. Effect of chilling hours before outplanting. End of growing season root collar diameter (RCD), height, needles per total stem units for flush 2 (N/Unit) and the ratio of branch height to apical meristem height (Final Ratio). Data represents tubelings exposed to 0, 400, or 600 hours of 3°C before outplanting at the VPI & SU Reynolds Homestead Research Center in Critz. Values within a column followed by the same letter are not significantly different at $\alpha=0.05$.

Chilling Hrs	RCD	Height	N/Unit Flush 2	Final Ratio
0 Hours	8.06 a	28.53 a	0.495 a	0.899 a
400 Hours	7.06 b	27.66 a	0.521 a	0.844 a
600 Hours	6.38 c	24.52 b	0.477 a	0.684 b
Overall Mean	7.16	26.90	0.499	0.809

The influence of chilling (decreasing final ratio) and daylength (increasing needles per total stem units) treatments implies that the seedlings lost apical dominance and produced shoots with fewer needles because of an interruption in the normal dormancy cycle of loblolly pine. Although loblolly pine is a recurrent flusher that exhibits free growth, seedlings are influenced by a yearly dormancy cycle.

It is hypothesized that seedlings exhibiting abnormal growth are responding to shortening daylength, with the apex approaching a relatively more dormant state. However, growing conditions were still favorable, allowing lateral branches to continue growth. Further, Martin (1987) indicates that high light, nutrients, and water decrease apical control. The application of shortened days to establish a bud and subsequent chilling may have "reset" the seedlings, or brought them back into phase prior to outplanting.

CONCLUSIONS

Growth abnormalities in winter planted loblolly pine seedlings may influence tree form as several terminals may compete for dominance, resulting in low forking. The development of abnormalities varied with planting site and, although not discussed, by family. The development of abnormalities is likely the result of an interruption in the normal dormancy cycle of loblolly pine. Treatments that increased the dormancy status of the seedlings prior to planting, including more severe hardening off, shortened days and subsequent chilling favorably influenced the number of needles per stem unit and the expression of apical control. These results indicate that 6 weeks of shortened days and 600 hours of chilling before outplanting, as well as a more severe hardening off period, favorably influence seedling form.

As growth differences and the expression of needleless shoots and apical dominance varied between the Appomattox and Reynolds Homestead planting sites, an additional topic that might be investigated is seedling response to dormancy treatments and subsequent outplanting on the Appomattox - Buckingham State Forest.

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GENOME SIZE, TRACHEID VOLUME, AND ENVIRONMENTAL FACTORS IN THE GENUS PINUS^{1/}

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Abstract.--Positive relationships have been reported between genome size and cell volume both in prokaryotes and eukaryotes. In six North American Pinus species, there appears to be a direct relationship between genome size and measured tracheid volume in greenhouse-grown seedlings. However, habitat precipitation seems to affect tracheid volume of trees grown in the field. Using genome size and habitat precipitation data of six Pinus species, tracheid volume in field-grown trees was estimated quantitatively. A theory is introduced for estimation of tracheid volume, and two terms, standard tracheid volume and tracheid volume change, are defined. Using an equation estimating tracheid volume derived from the six species, tracheid volume in field-grown trees was estimated in 15 North American Pinus species. Estimated tracheid volumes showed significant agreement with those calculated from tracheid dimensions obtained from the literature. Although this is a preliminary report, these results indicate that the theory might be effective for estimation of tracheid volume in North American Pinus species.

Keywords: genome size, Pinus species, estimation of tracheid volume, standard tracheid volume, tracheid volume change, environmental stress.

INTRODUCTION

Positive relationships between genome size and cell volume have been reported both in prokaryotes and eukaryotes (Price et al. 1973, Shuter et al. 1983, Cavalier-Smith 1985). Before examining the relationship between genome size and cell volume in the genus Pinus, a comprehensive data set of genome size was needed. Recently, genome size in 18 North American and one exotic Pinus species including two seed sources of P. taeda was measured by scanning Feulgen microspectrophotometry and laser flow cytometry (Wakamiya et al. 1993). The two methods showed a significant agreement with each other. In this paper, the relationship between genome size and tracheid volume is examined.

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Fiber dimension is an important characteristic of wood for the forestry industry. Factors affecting fiber dimensions have been studied, including types of indices related to the dimensions such as specific gravity (Zobel and van Buijtenen 1989). However, little interest has been given to the quantitative estimation for fiber dimensions. In this paper, one theory is introduced for estimation of tracheid volume, and is examined in North American Pinus species.

THEORY

According to Newton et al. (1993), tracheid dimensions from the literature appear to be directly related to genome size when a species is distributed in a region where the habitat lowest annual precipitation exceeds 800 mm. Habitat lowest annual precipitation (PRC) is the lowest annual precipitation within the natural distribution range of the species. Where PRC is less than 800 mm, tracheid dimension appears to decrease, compared with expected values from the relationship between genome size and tracheid dimensions. The decrease in tracheid dimension from expected values is negatively correlated to PRC. Therefore, it is hypothesized that the tracheid has an expected dimension if soil water is adequate. We used tracheid volume as a tracheid dimension in this study.

We introduce two terms, standard tracheid volume (V_t°) and tracheid volume change (ΔV_t). V_t° is defined as tracheid volume of pine trees grown under standard conditions (well-watered). ΔV_t is defined as the decrease in tracheid volume from V_t° . Therefore, tracheid volume (V_t) is defined as:

$$V_t = V_t^\circ + \Delta V_t. \quad (1)$$

In the genus Pinus, V_t° is hypothesized to be a function of genome size and ΔV_t to be a function of PRC (Newton et al. 1993).

MATERIALS AND METHODS

Tracheid volume

Assuming that tracheid shape is columnar, tracheid volume (V) was calculated as $V = \pi R^2 L$, where R and L are tracheid radius and length, respectively. In this paper, values of R were calculated from tracheid width (W) as $R = W / 2$.

Tracheid length is dependent upon age. Fibers appear to continue to grow for several decades (Zobel and van Buijtenen 1989). On the other hand, tracheid width can be assumed to be quite constant after one full growing season. There is a relationship between tracheid length and width which has been reported in the pulp sector of the forest industry, and tracheid length was estimated by that relationship (Newton et al. 1993). Tracheid width of stems for greenhouse-grown seedlings was measured and that for field-grown trees was obtained from the literature (Newton et al. 1993). Tracheid volume calculated from tracheid dimensions obtained from the literature is denoted as $V_{t, \text{field}}$.

Standard tracheid volume (V_t°)

Standard conditions for determination of V_t° are as follows. Seedlings were grown under well-watered conditions in a greenhouse equipped with an evaporative air conditioning system and shaded with 50% shade cloth. They were fertilized frequently and watered every other day. Fritted clay was used as the growth medium.

Two-year-old greenhouse-grown seedlings of six species (Table 1) were used for measurement of tracheid width. Using the measured tracheid width, tracheid volume (V_t) of the six species was calculated from the method previously described and was used as V_t° . Genome sizes were obtained from Wakamiya et al. (1993) and are shown in Table 1. Correlation and regression analyses were done to examine the relationship between genome size and V_t° in the six species.

Table 1. Genome size of six Pinus species used for determination of the standard tracheid volume (V_t°).

Range in U.S.	Species		Genome size [pg] ^{a/}
South-East	<u>P. virginiana</u>	Virginia pine	21.1
South-East	<u>P. taeda</u>	Loblolly pine	23.0
North-East	<u>P. strobus</u>	Eastern white pine	26.7
West	<u>P. radiata</u>	Monterey pine	24.3
West	<u>P. lambertiana</u>	Sugar pine	29.7
West	<u>P. monophylla</u>	Singleleaf pinyon pine	30.2

^{a/} Obtained from Wakamiya et al. (1993).

Tracheid volume change (ΔV_t)

Tracheid volume change (ΔV_t) was calculated as the difference between $V_{t, \text{field}}$ and V_t° . Correlation and regression analyses were done to examine the relationship between PRC and ΔV_t in the six species. PRC was obtained from Wakamiya et al. (1993).

Estimation of tracheid volume

Tracheid volume for 15 North American Pinus species was estimated using Eq. 1. Estimates of V_t were compared with values of $V_{t, \text{field}}$. Paired t-tests, correlation and regression analyses were used to substantiate the theory.

The 15 Pinus species used in the analysis were: P. virginiana, P. clausa, P. serotina, P. echinata, P. taeda, P. elliotii, P. strobus, P. radiata, P. attenuata, P. monticola, P. coulteri, P. torreyana, P. sabiniana, P. lambertiana, and P. monophylla.

RESULTS AND DISCUSSION

From the regression line where genome size was related to standard tracheid volume (V_t°) in the six Pinus species ($r^2 = 0.96$, $P < 0.1\%$), V_t° was described as

$$V_t^\circ = f(\text{genome size}) \quad (2)$$

where f denotes a function.

Figure 1 shows the relationship between PRC and ΔV_t . As previously suggested (Newton et al. 1993), $V_{t, \text{field}}$ decreased where PRC is less than 800 mm.

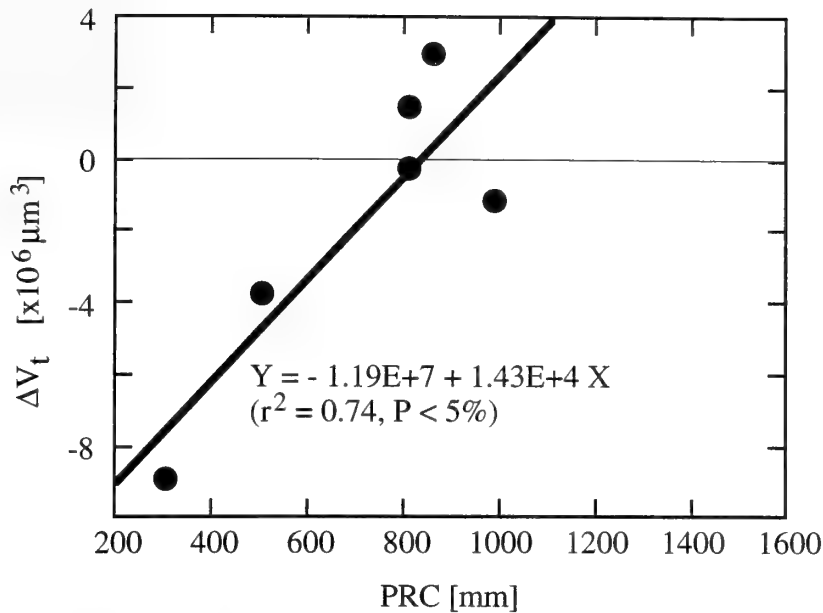


Figure 1. The relationship between the tracheid volume change (ΔV_t) and habitat lowest annual precipitation (PRC). At less than about 800 mm, tracheid volume decreases, compared with standard tracheid volume (V_t°). If tracheid volume calculated from tracheid dimensions obtained from the literature ($V_{t, \text{field}}$) is the same as V_t° , a plot is then shown by the thin line ($Y = 0$).

From the regression line describing the relationship of PRC to tracheid volume change (ΔV_t) ($r^2 = 0.74$, $P < 5\%$), ΔV_t was described as

$$\Delta V_t = f(\text{PRC}). \quad (3)$$

V_t obtained using Eqs. 1, 2, and 3 was compared with $V_{t, \text{field}}$. No significant differences between those values were shown by a paired t-test. Figure 2 shows the relationship between V_t and $V_{t, \text{field}}$. Both values were significantly correlated ($r^2 = 0.50$, $P < 1\%$) and showed significant agreement (intercept = -0.95; slope = 1.12) (Fig. 2). These results indicate the effectiveness of this theory for estimation of tracheid volume, even though collected data used for substantiating this theory were from a small number of species.

CONCLUSIONS

In North American *Pinus* species, we were able to estimate tracheid volume of trees grown in the field using genome size and PRC. However, genome size and PRC are not the only factors related to tracheid volume. It is commonly known that several environmental factors such as air temperature and physiological characteristics such as age and genetic traits such as coding genes also alter cell volume. Also, precipitation data and values of tracheid dimensions used in this study are very general. Intraspecific variation needs to be studied, and standard environmental conditions should be determined and defined for standard tracheid volume (V_t°). For more accurate estimation of tracheid volume, further investigations are needed.

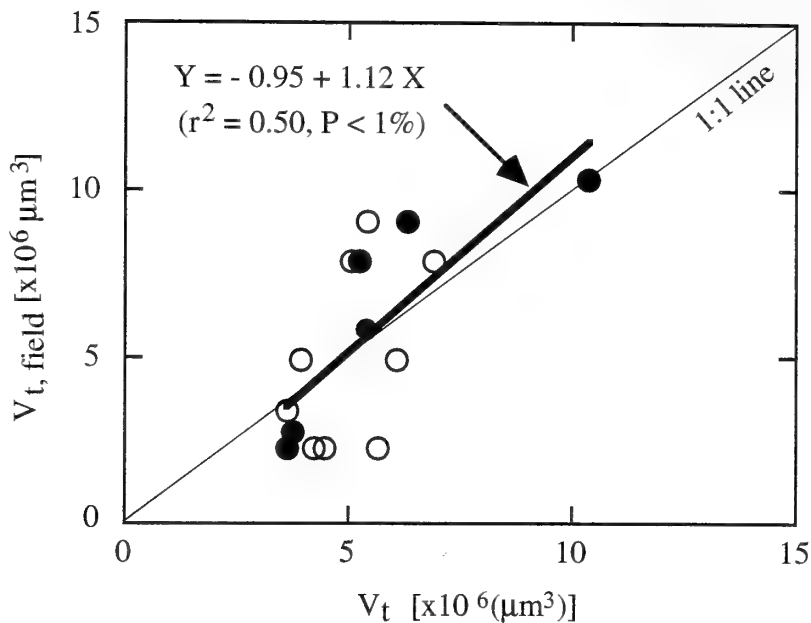


Figure 2. Comparison of estimated tracheid volume (V_t) and tracheid volume calculated from tracheid dimensions obtained from the literature ($V_{t, \text{field}}$) in 15 North American *Pinus* species. The solid line is a regression line for 15 species. The black circles show the six *Pinus* species used to determine ΔV_t listed in Table 1 and the white circles represent the remaining 9 of 15 species in the analysis.

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FUSIFORM RUST - A MODEL FOR MARKER ASSISTED SELECTION IN LOBLOLLY PINE?

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Abstract. Recent advances in DNA marker technology have enabled forest geneticists to better dissect the genetic bases for complex traits such as host pathogen interactions. Information from such experiments can be directly integrated into breeding programs, with the potential for significantly advancing the rate of gain in resistance breeding. Here we describe the steps involved in marker assisted selection, including hypothesis development regarding major gene presence and mode of action, mapping resistance loci with DNA markers via cosegregation analysis, and the subsequent use of this information for breeding purposes. This approach effectively integrates some of the recently developed tools of molecular biology with the more traditional quantitative genetics to solve problems in tree breeding that have not previously been considered tractable.

INTRODUCTION

The advent of more advanced DNA marker technology has led to widespread experimentation focused on dissecting the genetic basis of continuous traits in many plant and animal species. The seemingly common occurrence of a few loci of disproportionately large contribution to genetic variance (referred to hereon as 'major genes') has led to reconsideration of the use of such discrete markers for selection of continuously distributed traits in breeding programs. There are four stages involved in applying DNA markers to tree breeding (Figure 1): i) *identification* of traits and individuals that have major genes contributing to genetic variance; ii) finding associations between markers and major gene loci via *cosegregation analysis* of segregating populations; iii) biological *validation of the statistical associations* between markers and trait loci; and iv) *implementation* of this

information into existing breeding programs. In this paper we briefly outline each of these stages with particular reference to rust resistance breeding in loblolly pine (*Pinus taeda* L.).

Figure 1. Stages Involved in Marker Assisted Selection

Examination of Quantitative Data for Detection of Major Genes



Determine Appropriate mapping strategies and approach(es) to cosegregation analyses:

- mode of gene action
- oligogenicity profiles
- estimation of linkage parameters



**Confirmation/Validation of the biological basis for QTL
(field and greenhouse studies)**



Integration into breeding programs:

- gain estimation:
 - selection index approaches
 - independent culling
- qualitative approaches

Marker assisted selection (MAS) in forest tree breeding is potentially useful for a variety of reasons, including i) the potential for early selection based upon marker genotype rather than waiting up to many years before obtaining breeding values from the phenotype; and ii) the high levels of heterozygosity in many tree species as well as the relatively few generations of breeding means the likelihood of detecting meaningful associations is considerable (if trees do indeed have major genes).

Because of the high cost and relatively low throughput of the technology, experiments to detect marker-trait associations have to be carefully planned, therefore requiring the correct choice of trait, marker technique, and mapping

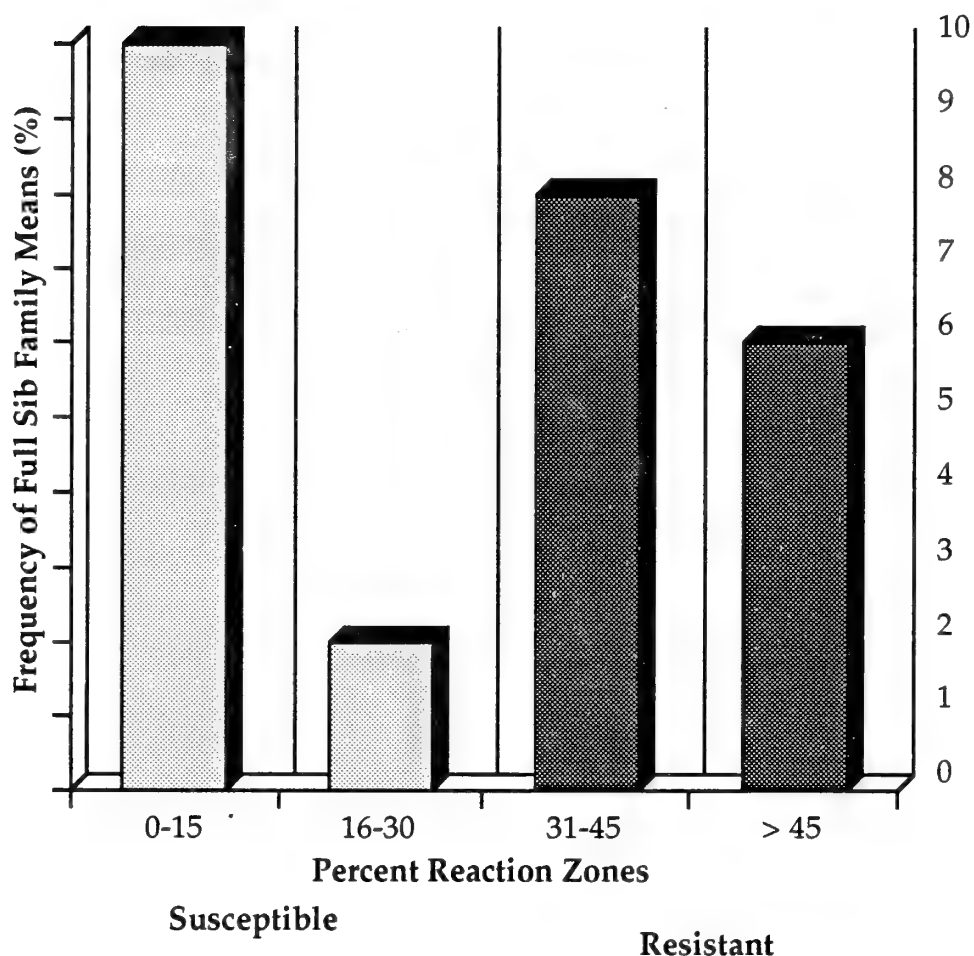
population(s). To date, most experiments involving DNA markers for dissection of quantitative traits are in the data collection phase. In choosing the appropriate trait, forest geneticists have considered high heritability an indicator of oligogenicity (few genes of large effect) - wood density is an example (Williams and Neale 1992). An alternative trait is disease resistance, not because of heritability, but rather the likelihood of major genes due to the interaction of multiple, but discrete, pathogenic and resistance mechanisms (Strauss et. al. 1992) - despite the lack of evidence for single gene defense mechanisms in the vast majority of forest tree pathosystems.

Resistance in loblolly pine to the causal agent of fusiform rust, *Cronartium quercuum* f. sp. *fusiforme* (Cqf), is a potential candidate for the presence of major genes, and ultimately the integration of marker information into existing breeding programs, not only because of the potential for major genes but also because of the development of early screening procedures for resistance. Such procedures can potentially be used for detecting candidate loci associated with field resistance. This in turn may ultimately reduce screening cost if markers are used as a substitute or complement to further greenhouse screening, as the costs of greenhouse screening may ultimately be greater than genotyping using DNA markers. This may also apply to specialist breeding programs such as seedling seed orchards.

THE FIRST STAGE: LOOKING FOR MAJOR GENES IN QUANTITATIVE DATA

The first stage in MAS is the examination of quantitative data for evidence of major gene segregation (Figure 1). Examination of data from partial diallels which have been screened for resistance has shown strong evidence for segregation of major genes in both slash pine (*Pinus elliotii*) (Kinloch and Walkinshaw 1989) and loblolly pine (M. Carson 1983 unpubl., Wilcox unpubl. data). In the studies involving loblolly pine, M. Carson found bimodal distributions of half and full sib family means for symptom types such as rough galls and reaction zones (Figure 2). Subsequent examination of the data showed an unexpected pattern when the more resistant genotypes were crossed with the less resistant clones (where resistance was based on half sib family means): full sib family percentages showing the resistant symptom type were equal to that of full sib families of two resistant clones, implying gene action is dominant, and few loci are involved in altering the threshold of resistance in the full sib families. According to expectations of a polygenic basis for resistance where gene action is additive (Carson and Carson 1989), one would not expect to see either bimodal distributions of full and half sib family means nor percentage infection of the high X low resistance families similar to that of high X high resistance families. Subsequent examination of four more partial diallels involving five or six clones suggests the pattern of dominant gene action and bimodal distributions of family means predominates: of the four diallels examined, eight symptom type/diallel

Figure 2. Rust Diallel: Distribution of Full-Sib Means for Reaction Zones



combinations have been examined to date: six combinations exhibit the aforementioned pattern with the other two combinations showing little or no segregation whatsoever. Two tester mating designs involving a larger number of clones have also been examined: one showing a similar pattern to the diallels, whereas the other showed a less clear pattern. Whilst statistical analysis of this data is required before we can be more confident that major genes for resistance are present, patterns evident in the diallels are strongly suggestive of few segregating major genes. We have therefore hypothesized that greenhouse resistance is oligogenic, where gene action is dominant rather than additive.

In all of the above experiments with loblolly pine, the pathogen source used was genetically heterogeneous, being derived from gall mixtures.

Although this data has assisted us in deciding upon resistance as a potentially oligogenic trait, it is by no means mandatory to have such data before choosing any given trait. Researchers working with other plant species are commonly finding major genes for a wide variety of traits including growth and yield characteristics (e.g., Stuber et. al. 1992) which were at one stage thought of as having too many potential component loci to be oligogenic. Whether or not this is the case for forest tree species is yet to be decided.

THE SECOND STAGE: FINDING ASSOCIATIONS BETWEEN TRAITS AND MARKERS

Mapping trait loci in highly heterozygous organisms of long generation length is by no means an easy task: standard approaches in annual crop species such as backcrosses and intercrosses utilizing isogenic lines are not usually possible in forest tree species. There are however a variety of approaches available to forest geneticists to map quantitative traits depending upon factors such as the nature of the mapping population (full or half sib), mode of gene action and marker technology used. Quantitative trait mapping strategies include the three generation cross approach (e.g. Williams and Neale 1992), within half-sib family mapping (O'Malley et. al. 1992, Wilcox et. al. 1992, Liu et. al. 1993), pseudo-testcross (Grattapaglia and Sederoff 1993) and utilization of interspecific hybrid crosses (e.g., Bradshaw unpubl.). Pseudo-intercross approaches have also been used to map traits in *Prunus persica* (Chapparro et. al., in press). In addition, for coniferous species, the above mapping strategies may be used in combination with haploid and/or diploid tissue for genotyping with DNA markers. In addition, for disease resistance mapping, it is necessary to control or eliminate heterogeneity in pathogen virulence as well as environmental variance.

Because forest pathosystems typically lack well defined (iso- or near-isogenic) lines of both host and pathogen, a combinatorial experimental design has been used to identify informative heterogeneous families. A putative heterozygous (*Rr*) mother tree was chosen (clone 10-5 - a resistant clone) , along with four of its half-sib progeny, where all five clones were crossed to a highly susceptible pollen parent (*rr*). The rationale for using the half sib progeny as parents is that the desired segregation may not occur if clone 10-5 is homozygous at the major gene locus for resistance. All of the resulting full-sib families have been challenged with inoculum from six single aeciospore lines (SALs) which vary in their virulence to the progeny of the mother clone. The desired SAL genotype is effectively homozygous for avirulence, as this will be the most informative given the putative *Rr* X *rr* host parental cross. Resistance has been scored for several greenhouse symptom types in the progeny of each family X SAL combination at three, six and nine months.

RAPD markers segregating in the haploid megagametophytes of all progeny are being used to construct RAPD linkage maps of the parent clone, 10-5, as well

as the half-sib progeny of 10-5. Maps will be used to find associations between markers and resistance that is maternally inherited (as we are using a putative susceptible homozygote pollen donor). Two SALs have been targetted: three and six month data indicate SALs NC 2-36 (50% expression of percentage galled symptom type at six months) and 2-40 (80% galled, therefore segregation of gall phenotypes) will be most informative for a range of symptom types. Maps for families A (full sib progeny of 10-5 X susceptible parent) and B are currently being constructed using haploid megagametophytes from the NC 2-36 SAL combination. These maps should be representative of each family regardless of pathogen inocula, hence detection of marker-trait associations for SAL NC 2-40 will entail genotyping the megagametophytes from this combination for markers approximately every 10 cM, rather than repeating the entire mapping exercise.

STEP 3: BIOLOGICAL VALIDATION OF ASSOCIATIONS BETWEEN MARKERS AND MAJOR GENE LOCI

For markers to be useful in breeding programs, validation of (statistical) marker - trait associations will be necessary. Validation may involve replicating experiments with different progeny from the same cross(es), testing in different environments, and in the case of long lived perennial species, at different ages. In our experiments, two validation steps are necessary. Firstly, validation of 'greenhouse' rust resistance should be possible by using related families, where the associations found in one family between a given chromosomal region and resistance to a given SAL (or set of SALs) should also hold for another family. However, even if resistance maps to different chromosomal regions for different families (locus heterogeneity), this does not necessarily invalidate associations. The second validation step will be to test associations derived from greenhouse screening in field experiments. Because the linkage maps we produce will be specific to individual clones, markers associated with resistance in a given family may not be linked to resistance in non-related families, therefore we are restricted to families that have been screened in the greenhouse *and* planted in field tests. Marker-resistance associations may be different for different families, thus may be of limited use only. However, use of clone 10-5 seedlots has been widespread in both greenhouse and field experiments, offering opportunity for validation in field experiments.

STAGE FOUR. INTEGRATION OF MARKER-TRAIT ASSOCIATION INTO EXISTING BREEDING PROGRAMS.

The ultimate use of marker-trait associations for breeders will be for selection of genotypes with superior breeding value. There are a variety of ways in which markers can be used for improvement of quantitative traits. In the case of rust resistance, marker-trait associations can be utilized in either a quantitative or qualitative manner. Some examples of both are outlined in this section.

With continuously distributed traits, quantitative approaches to gain estimation appear to be sufficiently adaptable to allow the evaluation of marker-trait information in breeding programs. For example, Lande and Thompson (1990) outlined the application of index selection theory to combined selection based on marker and phenotype. This approach has been used by Zhang and Smith (1992) to show combined marker and phenotypic selection will be more efficient than either alone in animal breeding programs. Simulations by Strauss et. al. (1992) for forest tree breeding suggested combined selection will be more efficient when within family heritabilities are low, and between family heritabilities are low to moderate. Use of index selection theory for threshold traits such as rust resistance has been developed (Danell and Ronningen 1981), as has theory for combining threshold and continuously distributed traits (Cue and Hayes 1985). It should theoretically be possible to evaluate gain and estimate index weights for both multitrait and family indices. A further approach may be two-stage selection, where only genotypes with (greenhouse screened) resistance loci are planted out for further resistance screening in genetic tests, effectively increasing selection intensity. A further use of marker information may be to select for resistance in situations where there is no variance in the trait: selection of individuals within families that have been screened for resistance in the greenhouse and also planted out in field tests is possible. In this case, selection for resistance can be made on individuals planted in field tests even if no resistance variation exists, by using marker genotype, whereas other traits may be selected based upon field information.

In contrast with continuously distributed traits, markers for resistance may also be used in a qualitative manner to obtain gain via the alignment of multiple defense mechanisms in a predetermined fashion. This approach utilizes the potential for markers in combination with histological and biochemical techniques, to dissect resistance into discrete mechanisms, each of which may be controlled by several loci. With this information, it may be possible to take a multiple population approach to breeding for resistance, where each population has all resistance mechanisms present in each family, but different populations having different loci controlling each mechanism. To offset pathogen evolution, the production populations would therefore be constructed of population mixes, rather than alone. This will approach potentially allows breeders to manipulate mechanisms and loci, whilst maintaining sufficient diversity to reduce risk of catastrophic losses due to pathogen evolution.

CONCLUSIONS

There are four stages involved in the using DNA markers for selection purposes, three of which are considered essential. The first stage is the selection of trait and segregating population for mapping. This stage may turn out to non-essential due to the presence of major genes for the majority of traits as well as

the high levels of heterozygosity at trait loci for forest trees. The next stages involve finding associations between trait and marker loci via cosegregation analyses; followed by biological validation of these statistical associations. The final stage is the implementation of this information in breeding programs, for which there are a variety of potential quantitative approaches. In addition, for more discrete traits (such as disease resistance), qualitative approaches to achieving gain are potentially possible.

Rust resistance has the potential to be a model trait for marker assisted selection, since seedlings resistance can be screened at six to nine months of age, and is potentially more cost effective to screen breeding populations with markers. Furthermore, resistance appears to be oligogenic - at least in greenhouse screening - and offers the potential for a variety of approaches to be employed when using marker information for selection.

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SESSION 4

Breeding and Progeny Testing

REALIZED GENETIC GAINS IN VOLUME, VOLUME PER ACRE, AND STRAIGHTNESS

IN UNROGUED ORCHARDS OF THREE SOUTHERN PINE SPECIES

T. La Farge ^{1/}

Abstract.--By means of Best Linear Prediction (BLP), breeding values were obtained for single-tree volume, volume per acre, and straightness for shortleaf pine (*Pinus echinata*), eastern white pine (*P. strobus*), and three breeding populations of loblolly pine (*P. taeda*) in unrogued clonal seed orchards. For the volume traits the gains ranged from a high of 31.2 percent to a low of -3.8 percent. For straightness the gains were more stable across breeding populations, ranging from a high of 29.7 percent to a low of 0.1 percent. Substantial gains can still be obtained for volume in those breeding populations with weak or negative gains for volume when selection is restricted to a few of the best families. This kind of strategy is being implemented in those breeding populations by means of forward selections that are currently in progress. Possible reasons for the variability of relative gains for volume and straightness are discussed.

Keywords: Best linear prediction, breeding population, unrogued clonal seed orchard, realized genetic gain.

INTRODUCTION

In the Tree Improvement Program of the Southern Region (Region 8), progeny testing, which was begun in 1978, includes measurements at ages 5 and 10. Volumes can not be estimated until age 10, since dbh is not measured until then. Likewise, straightness is also evaluated at that age. Since a large number of progeny tests have been evaluated at age 10 in several species and breeding populations within those species, it is now possible to estimate genetic gains for volume and straightness for these species and populations.

Although some thinning has been done in the clonal seed orchards in which these breeding populations are maintained, there has been essentially no roguing in these orchards. Hence, any genetic gains to be obtained thus far in the breeding program are dependent on the success of the selection intensities obtained in the original ortet selection process and can be evaluated by comparison with commercial or General Forest Area (GFA) check lots.

This paper will report the heritabilities and genetic gains for volume, volume per acre, and straightness in five breeding populations representing three southern pine species.

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MATERIALS AND METHODS

Species and Breeding Populations

The three species reported in this paper are shortleaf pine (Pinus echinata Mill.), eastern white pine (P. strobus L.), and loblolly pine (P. taeda L.). The Region 8 Tree Improvement Program divides southern pine species into separate breeding populations when information is available to delineate such populations. Hence, shortleaf pine and loblolly pine are so subdivided based on the Southwide Pine Seed Source Study (Wells and Wakely 1966). White pine comprises only one breeding populaion, since insufficient information is available on geographic variation in this species in the southern Appalachians to designate separate populations.

In this paper shortleaf pine Breeding Population 1 (BP1), located on the Ouachita and Ozark-St. Francis National Forests in Arkansas and Oklahoma, is the only population in shortleaf pine with sufficient test data yet available to allow a comprehensive analysis. White pine Breeding Population 1 is so designated because information at some future time may allow us to subdivide the species range in the southern Appalachians. Loblolly pine is subdivided into seven breeding populations, three of which are reported on in this paper. These are BP2, BP3, and BP5.

Breeding Population 2 is located in Louisiana and Texas on the Kisatchie National Forest and the National Forests in Texas. Breeding Population 3 comprises the National Forests in southern Mississippi. Breeding Population 5 comprises the Districts on the Sumter National Forest in the Piedmont of South Carolina and the Uwharrie National Forest in the Piedmont of North Carolina.

Progeny Test Design

The field designs of the progeny tests in all three species conformed to the standard randomized complete-block design used in the Region 8 Tree Improvement Program. In this design the tests are arranged in 10-tree row plots so that all plots within a block followed the same contour. Most tests contained five replicates, but some had either four or six replicates.

Breeding Population Size and Number of Tests

The numbers of clonal seed orchard parents, the numbers of commercial or General Forest Area (GFA) check lots, and the number of progeny tests in each breeding population are listed in Table 1. Since the number of 6 X 6 diallel crossing groups varied from test to test, those numbers are not listed. Although the attempt was made to include all crosses within a crossing group in the same set of tests for any one year, some crosses were tested in years different from the majority of crosses in that crossing group. In the present analysis, the effects of differing sites and years were minimized by analysis of all data as deviations from each test mean. However, this analysis did not include the effect of genotype x year of establishment interactions.

Table 1. The numbers of parents, progeny tests, and commercial (GFA) check lots comprising each breeding population in three southern pine species.

Species and Breeding Populations	Parents	Progeny tests	GFA check lots
	<u>Number</u>	<u>Number</u>	<u>Number</u>
Shortleaf Pine Breeding Population 1	78	12	1
White Pine Breeding Population 1	60	14	4
Loblolly Pine Breeding Population 2	61	8	1
Loblolly Pine Breeding Population 3	42	9	1
Loblolly Pine Breeding Population 5	51	13	1

Procedures Used

The results of these analyses were based on data from measurements made at ten years of age. The traits measured were total height to the nearest foot, dbh to the nearest inch, survival, and straightness based on an index estimated ocularly to produce scores from 1 (=very crooked) to 4 (=straight). Traits analyzed were cubic volume inside bark of entire stem from volume equations for each species and breeding population. The volume equations are listed in Table 2. Volume per acre was calculated by summing the individual tree volume per acre at 8 X 8-foot spacing and factoring in survival.

All three traits were analyzed by means of Best Linear Prediction (BLP). BLP has been utilized as a method of data analysis because of considerable imbalance in the data available in all five breeding populations. Except for the white pine progeny tests, the mating scheme for all breeding populations is a 6 X 6 diallel crossing group. The white pine tests are wind-pollinated tests. Each year full-sib and half-sib tests were planted at as many as five locations, but not all families could be planted at each location, and most diallel crossing groups were less than complete. Hence, the prospect of obtaining balanced ANOVAs and unbiased estimates of breeding values were formidable until the recent availability of BLP as a method of data analysis (White et al. 1986; White and Hodge 1989).

Previously La Farge and Gates (1991) discussed the application of BLP in obtaining breeding values for height and survival in shortleaf pine (Pinus echinata Mill.) in Arkansas. Since those methods of data analysis were also utilized for the present investigation, they will not be discussed in depth in this paper. However, a brief review of the procedures required for these analyses is appropriate. BLP involves the prediction of breeding values, and

the defining equation is $\hat{g} = C'V^{-1}y$, where C is a non-symmetric matrix which defines the genetic relationships between the observed full-sib family means at each site and the true yet unknown breeding values, g; V is a symmetric matrix which represents the variances and covariances between the observed phenotypic values; y is a vector of data representing observed deviations of the family means at each test location from the test location mean; \hat{g} = the breeding values to be predicted.

Generation of the second moments needed to construct the C and V matrices requires a combined ANOVA on the data. This was accomplished by means of the VARCOMP Procedure of the Statistical Analysis System for Personal Computers and the MIVQUEO Method (SAS 1987). The variance components generated by this procedure were then combined in appropriate equations to produce the second moments needed to construct the C and V matrices. These second moments were loaded into the C and V matrices by means of language provided in the SAS IML Guide for Personal Computers (1985).

Genetic gains for each trait in each breeding population in each species were calculated as:

$$\% \text{ Gain} = (\text{Parental mean} - \text{GFA mean}) \times 100 / \text{GFA mean},$$

where Parental mean = the mean of all parental breeding values in a breeding population, and GFA mean = the breeding value for the GFA check lot or the mean of the breeding values of more than one check lot.

Table 2. Volume equations used to calculate single-tree volumes for each species and breeding population.

Species and Breeding Breeding Population	Volume Equation
Shortleaf Pine BP1	Total Vol.(ib) = 0.00914 + (0.0019281*dbh*dbh*height) <u>1/</u>
White Pine BP1	Total Vol.(ib) = 0.74000 + (0.0027610*dbh*dbh*height) <u>2/</u>
Loblolly Pine BP2	Total Vol.(ib) = 0.03789 + (0.0020911*dbh*dbh*height) <u>3/</u>
Loblolly Pine BP3	Total Vol.(ib) = 0.03789 + (0.0020911*dbh*dbh*height) <u>4/</u>
Loblolly Pine BP5	Total Vol.(ob) = 0.00914 + (0.0019281*dbh*dbh*height) <u>5/</u>

1/ Smalley and Bower (1968). 2/ Lacher and Schlaegel (1983).

3/ Schmitt and Bower (1970). 4/ Schmitt and Bower (1970).

5/ Bailey and Clutter (1970).

Table 3. Individual and family heritabilities in three southern pine species for volume, volume per acre, and straightness.

	Heritability, h^2	
	Individual	Family
<u>Shortleaf Pine Breeding Population 1</u>		
Volume	0.16	0.74
Volume per acre	.04	.46
Straightness	.13	.77
<u>White Pine Breeding Population 1</u>		
Volume	0.04	0.58
Volume per acre	.02	.23
Straightness	.03	.57
<u>Loblolly Pine Breeding Population 2</u>		
Volume	0.21	0.79
Volume per acre	.04	.42
Straightness	.13	.73
<u>Loblolly Pine Breeding Population 3</u>		
Volume	0.12	0.73
Volume per acre	.07	.63
Straightness	.10	.69
<u>Loblolly Pine Breeding Population 5</u>		
Volume	0.05	0.57
Volume per acre	.01	.24
Straightness	.11	.70

Table 4. Realized genetic gains for three southern pine species for volume, volume per acre, and straightness.

Realized genetic gain, percent	
<u>Shortleaf Pine Breeding Population 1</u>	
Volume	31
Volume per acre	30
Straightness	15
<u>White Pine Breeding Population 1</u>	
Volume	8
Volume per acre	-3
Straightness	0
<u>Loblolly Pine Breeding Population 2</u>	
Volume	0
Volume per acre	-4
Straightness	27
<u>Loblolly Pine Breeding Population 3</u>	
Volume	8
Volume per acre	2
Straightness	6
<u>Loblolly Pine Breeding Population 5</u>	
Volume	23
Volume per acre	18
Straightness	15

RESULTS

Individual and family heritabilities for single-tree volume, volume per acre, and straightness for each breeding population are listed for each species (Table 3), and realized genetic gains are shown for each breeding population in each species (Table 4).

DISCUSSION AND CONCLUSIONS

The considerable variability in genetic gain values suggests that basing genetic gain on comparisons with GFA or commercial check lots may be misleading. For three of the breeding populations, we could just as easily make backward selections on the basis of comparisons with the breeding population means as with GFA check lots. For one thing, records no longer exist which show the numbers of trees or stands comprising each GFA check lot.

The gains in some breeding populations may be weak because seeds were collected from trees or stands of average or above-average growth rate or straightness. Likewise, the very strong gains shown for Shortleaf Breeding Population 1 and Loblolly Breeding Population 5 may be exaggerated by comparison with GFA checks from stands or trees that are below average for growth or straightness.

Strictly speaking, there is no F-test for significance of differences among breeding values for parents in BLP. However, the weakly negative genetic gains shown in Table 4 for volume per acre in white pine BP1 and loblolly pine BP2 and for volume in loblolly pine BP2 are probably not significantly different from zero. Hence, although these gains are somewhat erratic in that they show some variability among breeding populations, they are basically quite strong. Moreover, in all breeding populations there are many parental breeding values that are well above average. Hence, there is ample room for obtaining genetic gains when making forward selections both for volume growth and for straightness.

The variability of these gains among breeding populations is perhaps an indication that collection of seed for use as commercial check lots in progeny evaluation testing should sample stands throughout the area of a breeding population, not just in one or two convenient cutting units.

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SLASH PINE FAMILIES IDENTIFIED WITH HIGH RESISTANCE TO FUSIFORM RUST

C. H. Walkinshaw ¹

Abstract.--Fusiform rust readily kills slash pine, *Pinus elliottii* Engelm. var. *elliottii*. When the number of rust-infected trees is high, an investment is altered and fewer trees are available for harvest. As infection reaches 80 to 100%, the healthy trees appear to be a good source of rust resistance. This method of obtaining resistance from selections of such trees has been highly productive. In this study, we have tried an alternative way to identify resistance. We selected rust-free slash pines with good tree characteristics in National Forests where incidence of rust is low (10-30%). Open-pollinated progeny from these trees were inoculated at the Resistance Screening Center (RSC). Parents that had the most resistant progeny at the RSC were then crossed. Their control-pollinated progeny were tested at the RSC. The resistance of some of these was unusually high. Resistance to fusiform rust is relatively common in individual slash pines.

Keywords: *Cronartium quercuum* f.sp. *fusiforme*, selection for resistance to rust, *Pinus* diseases.

INTRODUCTION

Tree-improvement programs initially emphasized growth and form in the selection of southern pines. When rust had obviously become a problem in the 1940's onward, selection procedures aimed to identify trees with rust resistance. Although emphasis is now shifting back to growth and form, fusiform rust remains a serious problem in slash pine *Pinus elliottii* Engelm. var. *elliottii*. But the extensive studies of Sluder (1989 A and B) and others show that genes for resistance to fusiform rust are not difficult to transfer and many trees carry these genes.

Slash pines that are resistant to fusiform rust occur as individuals throughout the range. They are generally identified as asymptomatic trees on high-hazard sites. An example of such a site is given in Walkinshaw (1987). These rust-resistant trees have been useful in forest industry, state forestry, and Forest Service programs. However, many of the rust-resistant trees are slow growers.

In this study good phenotypes for growth and form were identified on low-hazard sites and rust resistance was identified by inoculation. The most resistant trees were crossed. Data in this paper show that these control-pollinated progeny are highly resistant. Moreover, they strongly suggest that high resistance to fusiform rust in slash pine is relatively common in trees on sites with low rust.

METHODS

General Procedure

Four steps were followed in this research:

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1. A total of 223 slash pines with good growth and form were selected from the National Forests in Region 8. Rust infection in the forests within the vicinity of the selected trees was approximately 30%.
2. Open-pollinated seeds of these 223 pines were tested at Asheville (RSC) and Gulfport.
3. The trees with the most rust-resistant progeny and other moderately resistant trees were crossed and the control-pollinated seed were tested at the RSC.
4. Control-pollinated seedlings without swellings after 6 months were planted in the National Forests.

Inoculation Designs

Open-pollinated seedlots at the RSC in Asheville, North Carolina, were tested in two replications of three trays of twenty seedlings per family. Control-pollinated seedlot tests were replicated three times with three trays of twenty seedlings per family. Replications were one week apart and freshly prepared inoculum was used. Trays were arranged in a non-ordered fashion within replications on the greenhouse benches.

The design for inoculations at Gulfport was identical to the description given by Walkinshaw and Bey (1981). These studies were on 211 crosses with forest industry trees.

Inocula Selection

Several hundred field isolates were used to obtain telia from water oak (*Quercus nigra* L.) or northern red oak (*Q. rubra* L.). These provided fusiform-rust inocula for open-pollinated seedlots. In contrast, the inoculum for the control-pollinated families was from one gall on a 10 year-old slash pine in Bogalusa, Louisiana. This gall was selected at random and adopted as the author's standard for ten years of screening at Gulfport, Mississippi and Asheville, North Carolina. To insure that this isolate provided virulent basidiospores for infecting the experimental seedlots, several resistant slash pine seedlots from the Cooperative Forest Genetics Research Program at the University of Florida were first inoculated. Infection was: M-707=42%, 52-56=89%, 6-56=70%, 179-55=33%, and 316-56=67%. A further test with 27 control-pollinated resistant trees at Gulfport insured that the isolate was pathogenic to a wide range of slash pines. Mean infection in this test was 56% and one-third of the putative resistant crosses had galls on more than 70% of their seedlings after 6 months.

Inoculations

Gravity ("tent"), airstream, and water suspensions were used to deposit basidiospores on needles and needle bases of 10-week-old seedlings in flats of soil or in containers. All procedures paralleled those reported previously (Walkinshaw and Bey 1981, Walkinshaw and Roland 1990).

Slash pine 211 was extensively inoculated at the RSC and at Gulfport in an effort to break down its resistance. The other highly resistant slash pines were inoculated only at the RSC.

Observations

Tree and gall traits were taken in detail but are not given in this paper. Resistance is defined as the absence of a visible swelling. Outplanted seedlings were selected from those with purple spots on the stems but no swellings. When this type ran out, those without spots and swellings were planted.

RESULTS

Resistance of Family 211

Family 211 x W had 23% infection in a tent test of 100 seedlings at Gulfport (Table 1). The mean percentage of seedlings with galls for the other Region 8 families in the inoculation was 80. Results of additional tests at Gulfport and Asheville with family 211 x W are given in Table 1. Note the large number of fungal collections that we used to inoculate the progeny of this slash pine family. The ability to resist so many rust isolates was unique among Forest Service and forest industry trees that we had tested.

Histological observations of family 211 that was inoculated at Gulfport showed a large variation in the amount of cambium that was invaded 21 days after inoculation. The range was 110 to 925 microns in stem-circumference for 211 crosses, while the susceptible control ranged from 594 to 1225 microns. The highly resistant University of Florida family (M-707) (Bey and Walkinshaw 1981) averaged 256 microns compared to 294 for the 211 crosses. All measurements of fungal growth were made on a highly virulent rust isolate.

Table 1. Fusiform rust incidence in open-pollinated slash pine progeny of family 211.^a

Inoculation code	No. field isolates in inoculum	Percentage of seedlings with galls
"Tent"	many	23
20.42	1 (x9)	32
114-81	150	40
302-82	1	5
3-8-1	30	0
3-8-2	30	3
3-8-3	30	1
108-3	30	24
110-83	30	4
111-83	30	9
113-83	30	20
115-83	30	37
303-85	1 (X12)	12
101-36	120	13

^a Two or three replications of 60 seedlings were inoculated with 20,000 basidiospores per ml. Test 20.42 and 303-85 used 9 and 12 single isolates, respectively. Susceptible checks had 65 to 88% of their seedlings with galls.

Parallel observations of resistant and susceptible lesion reactions on 211 x W showed a variation in fungal growth within and from the lesion. Periderm formation was rare compared to lesions on the highly resistant Florida trees

M-707 and 5-56. Reactions of family 211 were similar to those on Florida tree, 179.55 (Bey and Walkinshaw, 1981). In both families many of the lesions were so small that they were not visible to the unaided eye. Many of the successful infections in these two families developed from invaded needle trace tissue.

Resistance of Other Select Trees

In addition to family 211, several other highly resistant slash pine were identified among the 223 Region 8 selections. The ones used in the crossing experiment and their percentage with galls in the open-pollinated progeny were: 211=14%; 224=35%; 331=42%; 311=50%; 304=50%; and 310=37%. Susceptible check seedlots averaged 88% of their seedlings with galls. Infection of the resistant check was 68%.

Resistance of Crosses

Control-pollinated crosses between slash pine 211 and eleven slash pine families that were being studied at Gulfport were highly resistant at the RSC (Table 2). Infection of the crosses was much lower than in the resistant check families. The gall-free survivors at the beginning of the 12th growing season ranged from 5.0 to 5.7 inches d.b.h.

Table 2. Percentage of control-pollinated progeny of slash pine 211 crosses that were galled six months after inoculation.^a

Cross	Galled after 6 mos.	D.b.h. after 11 yrs. (in.) ^b
211 x 8-4	5	5.0
211 x A-20	8	5.3
211 x J-1-5	2	5.3
211 x JP-1	2	5.3
211 x H-28	0	5.4
211 x H-7	2	5.4
9-2 x 211	1	5.5
8-7 x 211	2	5.6
211 x 18-27	2	5.6
211 x 21-58	2	5.6
211 x 211-55	3	5.7

^a Two replications of 60 seedlings were run with each of two single gall isolates. Susceptible check averaged 70% and the resistant check had 55% infection.

^b D.b.h. for seedlings with stem purple spots but no galls that were planted in the DeSoto National Forest at a 10 x 10 spacing.

Progeny of crosses of Region 8 trees had high resistance when inoculated at the RSC (Table 3). Susceptible families 347, 244, 232, and 214 had 97 - 100% diseased. The RSC susceptible check had 85% infection. All crosses of the resistant trees had less infection than the RSC resistant control seedlot.

DISCUSSION

Our inoculations show that high resistance to fusiform rust exists in slash pines on low-hazard sites. Moreover, the resistance of tree 211 was effective against many field isolates of the rust fungus. The infection of progeny of trees 211, 224, 311, 310, 331, and 304 and others at the RSC was unusually low. The fact that the five susceptible seedlots ranged 85 - 100% is proof that the inoculation was effective. As a group these control-pollinated trees are the most resistant ones that I have tested in 25 years.

When one examines the 223 slash pine families that were tested at the RSC by the Forest Service, Region 8, it is interesting to

discover that approximately 1 tree of 25 good growers has good resistance. The other 24 trees are susceptible. This pattern appears in the good growers selected for potential rust resistance in the 1950's (Barber, 1961). Clone 243-56 of the University of Florida was one of the good growers with rust resistance when tested at Gulfport and Taylor County, Florida. Other good growers in the top 25, such as 60-56 and 73-56 were susceptible. This somewhat random relationship between growth and rust resistance suggests that the pressure to select for resistance was exerted in prior generations. The period of co-existence of the rust and its host (several million years) would have provided ample opportunity for selection.

Table 3. Low infection in control-pollinated crosses of select slash pines in the USDA Forest Service Region 8 Program.^a

Cross	Percentage of seedlings with galls
211 x 211	9
224 x 331	12
311 x 311	18
304 x 304	19
331 x 211	19
224 x 304	26
211 x 304	27
331 x 310	28
310 x 311	32
310 x 331	33
311 x 211 Seedlot A	38
311 x 211 Seedlot B	39
Resistant check (RSC)	47
Susceptible families	
RSC	85
Region 8A	100
Region 8B	99
Region 8C	97
Region 8D	97

^a Three replications of 60 seedlings were inoculated with a single virulent isolate of the rust fungus at a concentration of 20,000 spores per ml.

Microscopy of needle-base lesions on slash pine 211 was interesting. Lesions were unusually small and the fungus was killed before

a periderm was formed. When galls formed, they were similar in size to susceptible controls. These reactions were observed with nine different single-isolates. The ability of a slash pine to restrict fungal growth to a few cells after penetration is common to many of the University of Florida resistant families, e.g., 179-55. Family 211 exhibits reactions much like these and other Florida families but is more effective in killing the rust fungus in the outer cortex.

CONCLUSIONS

Individual rust-resistant slash pines commonly occur in the southern forest. These can be found on both high- and low-hazard sites. Asymptomatic trees on high-hazard sites have been excellent sources of resistance, but their growth and form can be poor. The alternative procedure that was used in this study led to rust-resistant selections that were suitable for planting in high-rust hazard areas. Gains in growth and form can be greater in low-to-moderate-rust areas by utilizing the best families even though they are more susceptible to rust.

In contrast to the pessimistic view in 1960-70, the present study, along with the thorough work of E.R. Sluder (1989 A and B), strongly suggests that obtaining slash pines with good growth and form that also resist fusiform rust is not difficult (Kinloch and Walkinshaw 1991).

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RESPONSES OF THREE TEXAS LOBLOLLY PINE FAMILIES TO
AECIOSPORE ISOLATES OF CRONARTIUM QUERCUM F.SP. FUSIFORME

E. G. Kuhlman¹

Abstract.--Identifying aeciospore isolates of the fusiform rust fungus with virulence toward resistance in specific pine sources is the first step in our effort to ensure that a variety of resistance types are present in the resistant population. Open-pollinated seed from three Texas trees in the USFS/Georgia Forestry Commission orchard that had demonstrated good resistance to composite isolates of the rust were challenged by 13 specific isolates of the rust from Texas, Arkansas, Louisiana, and Georgia. Nine months after inoculation frequency of galls differed significantly for family, isolate, and family x isolate interaction. Gall frequency varied from 31-80, 7-55, and 26-55% for the three families. Isolates from TX and AR were more virulent on two of these families than were isolates from GA and LA.

Keywords: Fusiform rust, Cronartium quercum f.sp. fusiforme, loblolly pine, resistance, virulence

INTRODUCTION

Resistance in loblolly (Pinus taeda L.) and slash pines (P. elliottii Engelm. var. elliottii) to fusiform rust disease needs to have a broad genetic base to ensure its stability against the very heterogenous causal fungus, Cronartium quercum (Berk.) Miyabe ex Shirai f. sp. fusiforme. Rust resistance in agronomic crops is often overcome by the development of virulent isolates of a fungal pathogen. In these crops, resistance is often controlled by single genes, and some isolates of the pathogen have compatible genes for virulence that enable them to attack an otherwise resistant host plant. A broad genetic base of resistance in southern pines should reduce the risk of a virulent strain of the pathogen that previously occurred at a low frequency becoming dominant and causing severe losses.

Virulent isolates of the pathogen are useful for identifying different mechanisms or genes for resistance in the host population. Isolates virulent toward resistant loblolly and slash pines have been identified (Griggs and Walkinshaw 1982; Kinloch and Walkinshaw 1991; Snow and Griggs 1980; Kuhlman 1989, 1990, 1992; Powers 1985; Powers and Matthews 1979, 1980; Powers et al. 1977, 1978; Snow et al. 1975, 1976; Walkinshaw and Bey 1981). Virulent isolates produce gall frequencies in specific resistant families 1.5-2.0 times

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those caused by most isolates (Kuhlman 1990). Five of 21 loblolly pine families inoculated with six virulent, single-gall aeciospore isolates were uniformly, highly resistant (averaged <30% of seedlings galled). The other 16 families varied widely in response to the isolates and usually were susceptible (>65% seedlings with galls) to at least one isolate (Kuhlman 1992). One of the highly resistant families, 152-60, was from a Texas Forest Service collection. Powers and Matthews (1979, 1980) reported that isolates from the geographic source of some resistant material were often more virulent on that source than were isolates from other geographic sources. Single-gall isolate TX-3 was more aggressive on progeny of two Texas trees than were single-gall isolates from Georgia or South Carolina (Powers and Matthews 1979). Therefore, isolates from Texas appeared to be a possible source of virulence towards resistance in trees from Texas.

The objectives of this study were: (1) to identify rust isolates that are virulent on progeny from one or more resistant loblolly pines from Texas; (2) to determine if isolates from Texas are more virulent on Texas sources than isolates from other regions; and (3) to compare the responses of progeny from three Texas trees to 13 rust isolates.

METHODS

Seed Sources

Half-sib progeny from three trees in the seedling seed orchard (SSO) of rust resistant loblolly pines developed by the Georgia Forestry Commission and the USDA Forest Service were selected for this study. These trees came from a mass collection from a Texas Forest Service seed orchard. Progeny of tree 152-60 have a disease ratio (DR) (=frequency of galls in test family/frequency of galls in a susceptible control) of 0.30 in several concentrated basidiospore spray (CBS) system inoculations with composite isolates of the rust fungus. When progeny of this tree were inoculated with six single-gall isolates of the rust, 21% developed galls (Kuhlman 1992). Progeny of tree 151-79 have a DR of 0.33, whereas those of 152-266 have a DR of 0.54 after inoculation with composite isolates of the fungus.

Rust Isolates

Thirteen aeciospore isolates were studied. Eleven were from single galls and two were composite collections from several galls in an area. Two single-gall isolates, 152-201 and 3327-13, were chosen as avirulent controls since each produced galls on only 17% of the seedlings of 152-60 (Kuhlman 1992). Those two isolates and another single-gall isolate (G741) were from Georgia. Five isolates from Texas included three from single galls (TX-3, SG-A, and SG-1), and two from composite collections (SAT and LAT). Of the other five isolates, three were from single galls in Arkansas (77A-1, 77A-6, and 77A-10) and two were from single galls in Louisiana (LLL-3 and LLL-7).

Study Design

Seeds were moist, cold stratified for 6 weeks before planting in vermiculite in germ trays. Seedlings were transplanted 20 to a flat in a mixture of gravel and Fafard II Mix. When the seedlings were 6 weeks old, eight flats of each source were inoculated in the CBS system with one of the isolates. Four flats of each source were inoculated with one of the isolates on one day and the other four flats of each source were inoculated on a second day. Flats were randomly assigned to isolate treatment, and the order of isolate treatment was random. After inoculation the flats were placed in a moist chamber at 20 C for 24 hours, then moved to a greenhouse for 9 months.

Each seedling was examined for the presence or absence of galls 3, 6, and 9 months after inoculation. At 9 months, lengths of galls on live seedlings and rust associated mortality (RAM) also were observed. Seedlings that had galls at 3 or 6 months and were dead at 6 or 9 months were designated RAM.

The data at 9 months were subjected to a factorial analysis of variance with the SAS GLM procedure. Treatments were compared with Tukey's Studentized Range (Tukey's) at the 0.05 level. To elucidate regional trends in virulence, the incidence of galls by isolates from each state for individual families were compared.

RESULTS AND DISCUSSION

Frequency of galls, RAM, and gall length were each significantly affected by pine family, isolate, and the interaction of family x isolate (Table 1).

Table 1. Sources of variation, degrees of freedom, mean squares and significance for gall frequency, RAM, and gall length.

Source	DF	Mean square		
		Gall frequency	RAM	Gall length
Pine families	2	0.79286 *** ¹	0.15944 ***	1325.4612 ***
Rust isolates	12	0.27215 ***	0.13250 ***	279.6769 ***
Fam x isolate	23	0.13014 ***	0.03686 ***	118.9067 *
Error	246	0.01158	0.01186	65.0282

¹ ***=P<.001 *=P<0.05

Families varied significantly in the frequency of galls after inoculation with the 13 isolates (Table 2). Family 151-79 had the largest range in susceptibility from 31% with LLL-7 to 80% with 77A-1. Family 152-60 had the most resistant responses; six isolates produced infections on less than 25% of

the seedlings. The highest virulence toward 152-60 was 55% by isolate TX-3. Although family 152-266 had the least variation in response to the 13 isolates, the amount of infection with LLL-3 was more than twice that with SG-A and TX-3. Thus, on each of the three families, some isolates caused infections at least twice as frequently as other isolates.

Unique reactions of individual families to specific isolates led to a statistically significant family times isolate interaction. Note, for example, the gall frequency in families 151-79 and 152-60 inoculated with LAT (60 and 46%, respectively) and 77A-10 (38 and 23%, respectively). Incidence decreases by over 20%, whereas family 152-266 had 31% galls with LAT and 51% with 77A-10. Isolates 77A-6 and SGA had similar gall frequencies on families 151-79 and 152-60, but family 152-266 had 49% galls with 77A-6 and only 26% with SG-A.

Table 2. Frequency of seedlings with galls in three loblolly pine families nine months after inoculation with each of 13 single-gall isolates of *Cronartium quercuum* f.sp. *fusiforme*.

Isolate	State of Origin	Composite(C) or Single gall(S)	Family		
			151-79	152-60	152-266
77A-1	AR	S	80 a ¹	52 a	-
SAT	TX	C	76 ab	48 a	39 abcd
SG-A	TX	S	65 ab	53 a	26 d
TX-3	TX	S	62 ab	55 a	27 d
SG-1	TX	S	62 ab	51 a	31 bcd
77A-6	AR	S	60 b	41 ab	49 abc
LAT	TX	C	60 b	46 a	31 cd
77A-10	AR	S	38 c	23 bc	51 ab
LLL-3	LA	S	37 c	11 c	55 a
3327-13	GA	S	36 c	7 c	37 abcd
G741	GA	S	36 c	16 c	45 abcd
152-102	GA	S	33 c	22 bc	29 d
LLL-7	LA	S	31 c	13 c	35 bcd

¹Within columns, numbers followed by the same letter do not differ significantly (Tukey's = 0.05).

Isolates from Texas and Arkansas were more virulent on families 151-79 and 152-60 than were isolates from Louisiana and Georgia, producing 1.5-4.0 times as many galls (Table 3). Family 152-266 had more variation in response to the individual isolates and did not have significant differences in average infection among isolates from the four States. The results with the former two families support the observations of Powers and Matthews (1979) that isolates from Texas are more virulent on Texas sources, but also provide evidence of variations among rust isolates from Texas.

Table 3. Association of family susceptibility with state of origin of the rust isolates.

State of Origin of Isolates	Isolates (No.)	Family		
		151-79	152-60	152-266
Texas	5	65 a ¹	51 a	31 a
Arkansas	3	54 a	38 a	50 a
Louisiana	2	33 b	12 b	40 a
Georgia	3	35 b	15 b	37 a

¹Within columns, numbers followed by the same letter do not differ significantly (Tukey's=0.05).

Rust associated mortality of seedlings was affected by family, isolate, and the interaction of family by isolate. Family 151-79 had the most RAM just as it had the highest frequency of seedlings with galls. However, in all three families the isolate causing the highest frequency of galls did not cause the highest frequency of RAM (Table 4). Isolate TX-3 produced the highest frequency of galls on family 152-60 but the 14% of seedlings with RAM is near the mean for this family. Similarly isolate LLL-3 produced the highest frequency of galls (55%) on family 152-266, but the lowest frequency of RAM (6%) on the same family. Also on family 152-266, isolate 77A-10 produced 51% galls and the highest RAM of 29%.

Family 151-79 had the greatest average gall length in addition to having the greatest frequency of galls and RAM. However, family gall length response to isolates was independent of the gall-frequency and RAM responses (Table 5). Family 151-79 inoculated with isolate 3327-13 had a low frequency of galls, but the galls that formed were the longest in this family. Also, on family 151-79, isolate SG-1 produced many galls, but the galls were short. Similar variations occurred with the other two families. Isolates LLL-3 and LLL-7 were avirulent to family 152-60 but they had the longest and shortest galls, respectively, for this family.

CONCLUSIONS

Inoculation of three Texas families with isolates of the fusiform rust fungus from four States resulted in considerable variation in infection suggestive of three different types of resistance. Isolates 77A-1 and SAT from Arkansas and Texas, respectively, were highly virulent on family 151-79. Texas isolates were most virulent on families 151-79 and 152-60, but a Louisiana isolate produced the most infection on family 152-266. RAM and gall length appeared to be independent of incidence of galls.

Table 4. Frequency (%) of rust associated mortality among seedlings in three loblolly pine families after inoculation with each of 13 single-gall isolates of Cronartium quercuum f.sp. fusiforme.

Isolate	Family		
	151-79	152-60	152-266
SG-A	37 a ¹	30 a	15 ab
LAT	33 ab	13 bc	8 b
SG-1	31 abc	25 ab	13 ab
77A-1	28 abcd	22 ab	-
SAT	26 abcd	15 abc	11 ab
77A-6	22 abcde	10 bc	21 ab
TX-3	17 abcde	14 bc	9 ab
LLL-7	12 bcde	0 c	12 ab
77A-10	10 cde	4 c	29 a
152-102	9 cde	5 c	7 b
G741	8 cde	2 c	7 b
3327-13	6 de	1 c	6 b
LLL-3	3 e	0 c	6 b

¹Within columns, numbers followed by the same letter do not differ significantly (Tukey's=0.05).

Table 5. Average length of galls among seedlings in three loblolly pine families after inoculation with each of 13 single-gall isolates of Cronartium quercuum f.sp. fusiforme.

Isolate	Family		
	151-79	152-60	152-266
3327-13	38.0 a ¹	19.9 ab	26.7 ab
LLL-3	36.1 ab	32.0 a	31.5 a
SAT	33.5 ab	25.3 ab	21.4 ab
77A-1	32.5 ab	23.6 ab	-
TX-3	31.4 ab	27.7 ab	18.0 b
77A-6	31.3 ab	30.2 a	31.2 a
G741	30.7 ab	17.7 ab	29.5 ab
152-102	30.3 ab	24.9 ab	24.1 ab
LAT	30.1 ab	22.8 ab	17.9 b
77A-10	28.7 ab	30.8 a	25.4 ab
SG-A	28.6 ab	24.4 ab	17.4 b
LLL-7	23.5 ab	13.1 b	21.5 ab
SG-1	22.1 b	18.8 ab	20.3 ab

¹Within columns, numbers followed by the same letter do not differ significantly (Tukey's=0.05).

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GENETIC RESISTANCE TO TERMINAL WEEVIL ATTACK IN SITKA SPRUCE

J.N. King and C.C. Ying ¹

Abstract. -- The terminal or white pine weevil is an extremely damaging pest to Sitka spruce plantations in the Pacific Northwest and British Columbia. Fifteen-year records of weevil attack in Sitka spruce provenance trials in B.C. show marked differences in attack between provenance sources. Clonal re-testing of individuals selected from these provenance trials confirms this result. 90% - 100% of ramets from coastal fog-belt clones are attacked compared to some clones from the Georgia Lowlands / Puget Sound Basin that show no attack. Putative mechanisms to this genetic resistance have been identified and methods of deploying such resistance are discussed.

Keywords: Sitka spruce, terminal weevil, insect resistance, provenance variation.

BACKGROUND

Sitka spruce, Picea sitchensis (Bong) Carr., is a very valuable timber species in coastal British Columbia and the Pacific Northwest. It is a very vigorous growing species and its large size yields a high degree of clearwood. The high strength to weight ratio of the wood was used during the second world war for aircraft construction. Sitka spruce's wood properties and high yields have produced some of the highest stumpage values in British Columbia often twice the provincial average. Because of its value it is a highly desirable plantation species in some European countries; however planting of Sitka spruce in British Columbia and throughout the Pacific Northwest is very limited due to the destructive damage of the white pine weevil (Pissodes strobi Peck). The weevil damages the terminal leader after ovipositing in the bark at the top of the previous years terminal. Larvae from those eggs mine downward severing the cambial layer in the elongating terminal shoot. The leader droops and eventually dies. Repeated attack results in stunted deformed and outcompeted trees which rapidly become unmerchantable and eventually die (Alfaro 1982). In B.C. planting requirements for Sitka spruce are only about 1,000,000 per year this is mainly on the Queen Charlotte Islands, the North Coast, North Vancouver Island or outer parts of the coast that are affected by sea fogs. In these areas the weevil hazard is low or in the Queen Charlotte Islands is non-existent. Control techniques

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such as shading, leader clipping, insecticides or biological control agents have not proven very effective or practical (Stiell and Berry 1985, Alfaro and Omule 1990).

One of the most interesting promises in the control of this pest is genetic resistance. Genetic resistance was first indicated in species and cross species hybrid trials in Oregon and Washington (Mitchell et al. 1990). Lutz spruce, or the natural Sitka/white spruce hybrid showed markedly less attack - 9:56 trees predicted to be attacked were, compared to 134:117 predicted with pure Sitka spruce. Growth of the hybrid was very variable with some individuals exhibiting growth rates as good as pure Sitka. More valuable genetic resistance has emerged from an investigation of provenance trials established by the B.C. Forest Service. Trial sites, on 14 locations involving 43 provenance sources (some of them organized by the International Union of Forest Research Organizations, IUFRO), show some sources with a high degree of resistance to this damaging pest. Resistant provenance sources are from areas classified as "high weevil hazard" and include the drier areas in the rain shadow of East Vancouver Island and the Fraser River valley. This resistance is very marked in the provenance trials with a high degree of attack on the outer coast/fog belt sources compared to relatively little attack on resistant sources - less than one-quarter of resistant source trees attacked compared to other sources and less than half the average number of attacks per tree (Alfaro and Ying 1990, Ying 1991). In order to test the effectiveness of selection for resistant trees a clonal test was established using grafts from resistant and susceptible trees from the provenance trials (Ying 1991). The clonal trial repeated the results of the provenance trial. Clones from resistant provenance clones have 90% of grafts unattacked (Ying 1991) with some clones having all 16 ramets of the clone showing no attack at all; whereas over 95% of the susceptible source material has been attacked. Hybrid source material shows 50-60% of grafts not attacked, much better than susceptible sources compared, but not as good as resistant provenance sources.

Putative Resistant Mechanisms

These results are very dramatic in the field and have brought a lot of research activity by entomologists at Simon Fraser University in Vancouver and Forestry Canada, Pacific Forestry Centre in Victoria B.C.. Investigations have focused both on the reaction by the tree to the weevil attack and the reaction brought about in the weevil to its activities on the tree.

In reaction to the attack by weevils, resistant sources show resin ducts that are approximately twice the diameter of susceptible sources and perhaps more importantly resistant sources have significantly more outer resin ducts than those from susceptible provenance sources (Tomlin and Borden, MS subm). Resin is considered to be a primary defense mechanism for conifers, so having more and larger resin ducts would presumably contribute to

resistance. Also the existence of large numbers of these outer layer resin ducts may be associated in two other ways with resistance. Outer resin ducts produce traumatic resin which is known to contain higher amounts of defensive compounds, and they are the first line of defense encountered by weevils feeding or ovipositing on trees and thus can encumber their actions (Tomlin and Borden, MS subm).

In the reaction bought about in the weevil investigators have shown that weevils feeding on resistant source trees show a high incidence of absorption of the developing eggs back into the ovary walls (T. Sahota, Forestry Canada, pers. comm.). These efforts in discovering the underlying mechanisms to this genetic resistance offers great promise.

BREEDING AND DEPLOYMENT OF RESISTANCE

Incorporating Resistance Mechanisms into the Breeding Population

In our population improvement strategy with forest trees using recurrent selection for general combining ability we have not needed to know the underlying physiological mechanisms behind the traits we select for, instead we have relied on a quantitative genetic model which has worked very well. An eyes open approach with an understanding of how the mechanisms work and the genetics behind these mechanisms of resistance is especially important where the weevil will have its own genetic potential to counteract resistance mechanisms placed before it.

There is indication already that some of this resistance is under additive genetic control (Kiss and Yanchuk 1991). But we hope to understand more about the genetics to this resistance using both classical crossing experiments and associations with molecular markers. If we can obtain a clear understanding of the mechanisms and the gene action behind the mechanisms then a breeding population can be established with the resistance mechanisms and the different loci controlling these mechanisms applied as a matrix. This matrix approach which multi-layers the mechanisms and their underlying genetic controls will provide a more formidable resistance for the insect to overcome (S. Carson, NZ FRI pers. comm.).

Deployment of Resistant Material

We are fully aware now that insect pests are very resilient in overcoming resistance that has been placed before them. In the past important resistance has been squandered by not having a clearer understanding of the genetic and ecological interaction between host and pest. A clear understanding of the mechanisms and their genetic control will be required to apply this resistance matrix but just as important as this genetic resistance will be the silvicultural deployment of Sitka spruce. Large clearcuts and

spruce monocultures will just provide a smorgasbord for the weevil that will invite the insect to overcome any resistance that is placed before it. It is important that the genetic resistance that has been found not be squandered but used with appropriate silviculture to once again place Sitka spruce as a valuable commercial species in the Pacific Northwest.

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GROWTH AND STEM SINUOSITY OF DIVERSE PROVENANCES OF THREE-YEAR-OLD LOBLOLLY PINE

S.E. McKeand and J.B. Jett¹

Abstract.--Stem height and sinuosity of stem and of branches were measured at age three in a trial established in southwest Georgia with 13 to 16 OP families from each of four provenances of loblolly pine (*Pinus taeda* L.). Families from the Gulf Hammock, FL provenance were taller than families from the Atlantic Coastal Plain, Lower Gulf, and Middle/Upper Gulf provenances. Sinuosity also differed significantly by provenance with the fastest growing provenances having the most sinuous stems and branches.

The unfavorable correlation between growth and sinuosity at the provenance level was not evident among families within provenances. Apparently, the mechanisms causing the faster-growing provenances to be more sinuous were not the same at the family level within provenances. We hypothesize that the large differences in the length of the growing season for trees from the different provenances are partly responsible for the differences in sinuosity. Trees from southern regions grew longer than trees from the northern regions and possibly were not as lignified, increasing the likelihood of sinuosity.

Keywords: Breeding, genetic gain, *Pinus taeda* L., stem form, wood specific gravity

INTRODUCTION

The influence of provenance or seed source on stem form, notably straightness, has been well documented for numerous species (Zobel et al. 1987). Frequently, poor form results from the use of an incorrect seed source or provenance where a species is used as an exotic. At times, the poor form or straightness can be so extreme as to render the trees basically unusable.

Less well understood is the influence of provenance on stem form for seed movements of a less dramatic nature. Dietrichson (1964) linked latewood formation, tree heights, autumn frosts, spring frosts, stem straightness and wood lignification to provenances of Norway spruce (*Picea abies* (L.) Karst.) in Europe. Duration of growth was correlated with frost damage, latewood formation and ultimately with straightness. Latewood formation was negatively correlated with straightness with trees appearing more crooked as latewood percentage decreased. Even more significant was incomplete lignification which resulted in trees being more crooked.

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In loblolly pine, Jett et al. (1991) reported that trees from southern provenances such as Livingston Parish, LA and Florida consistently displayed low specific gravity when compared to trees from more northern sources. We postulated that the lower specific gravity of these sources might be explained by differences in patterns of shoot elongation. Zahner (1962) reported that latewood formation was not initiated until stem elongation ceased. If the southern sources grew longer than those of more northern seed lots, high density/high strength latewood would not be formed until later in the growing season, and lower wood specific gravity would result. However, Jett et al. (1991) made no attempt to evaluate growth phenology to determine the relationship of growth of these southern sources to their specific gravity. Subsequent observations of the same study trees indicated that seed source was beginning to have an impact on tree form consistent with the reports of Dietrichson (1964). This study was undertaken to determine the relationship between stem crook (sinuosity) and tree height both between and within four loblolly pine provenances.

MATERIALS AND METHODS

Seedlings originating from open-pollinated seeds of 13 to 16 families from each of five provenances in the southeastern United States were planted in a trial that was initially used for an early selection study (see McKeand and Bridgwater 1993 for details). Families from four of the provenances -Atlantic Coastal (ACP), Gulf Hammock (GH), Lower Gulf (LG), and Middle/Upper Gulf (MUG)- were assessed for the current study. Seedlings were grown in RL Super Cells^R (164 cc) in a greenhouse in Raleigh, NC until they were outplanted in March 1989 near Georgia-Pacific's (G-P) nursery at Cedar Springs, GA and at International Paper Company's (IPCo) Southlands Experiment Forest near Bainbridge, GA (Figure 1). A randomized complete block design with 36 blocks of single-tree plots of 72 families was initially used at each location. Thus, a total of 72 seedlings were planted per family. The trees were planted at a spacing of 1.3 x 1m at G-P and 1m x 1m at IPCo to minimize block sizes. No cultural treatments were imposed on the trees except that tip moths (*Rhyacionia* sp.) were controlled with periodic insecticide applications, and competing vegetation was controlled with periodic herbicide applications.

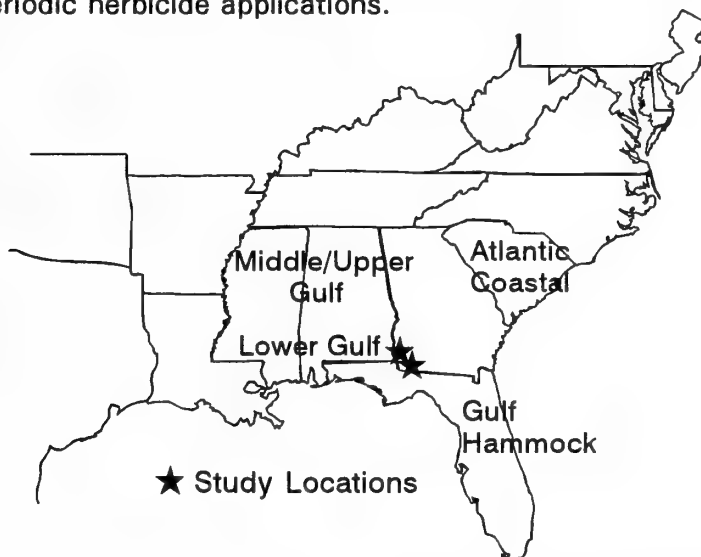


Figure 1. Map of the southeastern United States showing the general location of the four provenances and the location of the two field sites.

In February 1991, the study was thinned to allow for assessment of wood properties in subsequent years (see Anonymous 1993 for details). All trees from the Marion County, FL provenance were cut. Half the trees from the other four provenances were cut to leave one tree in each adjacent pair of blocks (e.g. original blocks #1 and #2 were considered as one block). Each family was now represented by 18 trees at the G-P site and 16 trees at the IPCo site (four of the original blocks at IPCo were dropped).

In 1993 at age 3 years, stem height, number of growth cycles (flushes), and sinuosity of the stem and of the branches were measured. Sinuosity was assessed on a three point scale: 1 = none, 2 = minor, 3 = major sinuosity. Other stem straightness defects such as crooks from broken leaders or tip moth damage were not assessed.

Family and provenance means across the two planting sites were calculated for each trait. All statistical analyses were conducted using the GLM and VARCOMP (Type I sums of squares) procedures in SAS (SAS Institute Inc., 1985). To estimate the significance levels for provenance effects in the analyses of variance (Table 1), an approximate F-test (Satterthwaite, 1946) was used. Genetic and environmental components of variance were estimated for each provenance separately. The variance among open-pollinated families within each provenance was assumed to estimate 1/4 the additive genetic variance (Falconer, 1989), and individual tree heritabilities for each provenance were calculated as:

$$h^2 = \frac{4 \sigma_F^2}{\sigma_F^2 + \sigma_{FL}^2 + \sigma^2}$$

where: σ_F^2 = variance among families
 σ_{FL}^2 = variance due to family by location interaction
 σ^2 = variance among trees within a family within a location

Standard errors of each h^2 estimate were calculated using methods of Becker (1984).

Table 1. Form of the analysis of variance used in the overall analyses.

Source	deg. of freedom	Expected Mean Squares ¹
Location	1	
Blocks(L)	32	
Provenance	3	$\sigma^2 + b\sigma_{L \times F(P)}^2 + lb\sigma_{F(P)}^2 + f\sigma_{B(L) \times P}^2 + bf\sigma_{L \times P}^2 + lbf\theta_P^2$
LxP	3	$\sigma^2 + b\sigma_{L \times F(P)}^2 + f\sigma_{B(L) \times P}^2 + bf\sigma_{L \times P}^2$
B(L)xP	96	$\sigma^2 + f\sigma_{B(L) \times P}^2$
F(P)	54	$\sigma^2 + b\sigma_{L \times F(P)}^2 + lb\sigma_{F(P)}^2$
LxF(P)	54	$\sigma^2 + b\sigma_{L \times F(P)}^2$
B(L)xF(P)	1666	σ^2
<hr/>		
Corr. total	1909	

¹ Provenances were considered as fixed effects, all others as random effects.

RESULTS AND DISCUSSION

At the end of three growing seasons in the field, overall mean height was 389cm, and 54% of the trees displayed some degree of stem sinuosity. Trees from the Gulf Hammock provenance were the tallest, followed by Atlantic Coastal, Lower Gulf, and the Middle/Upper Gulf. Provenance rankings for number of growth cycles and stem and branch sinuosity (Table 2) were the same as for height.

There was a relatively strong unfavorable correlation ($r_F = 0.58$) between height and stem sinuosity when families from all four provenances were compared. We hypothesize that the large differences in the length of the growing season for trees from the different provenances (e.g. Perry et al. (1966)) are partly responsible for the differences in sinuosity. If trees from southern regions grew longer than trees from the northern regions, it is possible that the southern trees were not as lignified as the northern trees, increasing the likelihood of sinuosity.

Within each provenance, there was moderate to strong genetic variation for growth and sinuosity traits (Table 3). The large family differences for stem height within each provenance was not significantly correlated with stem or branch sinuosity. Apparently, the mechanisms causing the faster-growing provenances to be more sinuous were not the same at the family level within provenances. We suspect that there is much less variation in the length of the growing season within each provenance as compared to among provenances. In a separate study, families of loblolly pine from a relatively narrow geographic range varied in height due to both duration of the growing season and rate of growth during the season (Bridgwater 1990). If stem sinuosity was mainly caused by poorer lignification because trees grew longer, a lower correlation between height and sinuosity within provenances would be expected.

Table 2. Provenance means for traits measured at age 3 years in the field.

Trait	Atlantic Coast (16 fam)	Gulf Hammock (15 fam)	Lower Gulf (14 fam)	Middle/Upper Gulf (13 fam)
Stem Height (cm)	397 ^b	437 ^a	367 ^c	347 ^c
Number of Growth Cycles	4.58 ^{ab}	4.76 ^a	4.31 ^{bc}	4.15 ^c
Stem Sinuosity Score	1.68 ^{ab}	1.79 ^a	1.60 ^b	1.47 ^c
% Sinuous Stems	57.3 ^{ab}	65.8 ^a	54.4 ^b	40.9 ^c
Branch Sinuosity Score	1.83 ^{ab}	2.01 ^a	1.67 ^b	1.56 ^b

Means within a row followed by the same letter are not significantly different ($p \leq .05$).

Table 3. Individual-tree narrow-sense heritabilities (standard errors in parentheses) for traits in different provenances.

Trait	Atlantic Coast	Gulf Hammock	Lower Gulf	Middle/Upper Gulf
Stem Height	0.53(.20)	0.40(.18)	0.49(.21)	0.81(.30)
Number of Growth Cycles	0.25(.13)	0.36(.16)	0.24(.13)	0.36(.18)
Stem Sinuosity Score	0.24(.12)	0.20(.11)	0.32(.16)	0.35(.18)
Branch Sinuosity Score	0.21(.11)	0.32(.15)	0.27(.14)	0.54(.23)

The genetic correlation between branch and stem sinuosity within each provenance was essentially perfect ($r_G = 1$). If a family tended to display sinuous branches, it also had sinuous stems. This is an important relationship for breeders, since branch sinuosity is displayed more commonly than stem sinuosity (Table 2). If a tree displays sinuous branches but not a sinuous stem, there is still a strong likelihood that its progeny will have sinuous stems.

While there are potential gains in growth from utilizing southern coastal sources of loblolly pine, the associated risk of reducing stem quality and also wood specific gravity (e.g. Byram and Lowe 1988, Jett et al. 1991) must be recognized. We are continuing to evaluate the present study to determine the relationships among growth traits, stem form, wood properties, and phenology. We hope to determine why relationships may differ among the traits depending upon the origin of the families.

ACKNOWLEDGEMENTS

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Abstract.--The utility of PC-based computer simulation holds promise as an effective tool for answering questions about genetic diversity in forest tree species. NATGEN is a prototype educational software package that combines management objectives, silvicultural prescriptions, diameter growth projections, and genetic parameters for one quantitative trait. The targeted species during development was northern red oak (Quercus rubra L.). Preliminary results of repeated diameter limit cutting (18 inches and above) for a population of size 100, indicated changes in desirable gene frequencies from 0.5 to 0.01, by 560 years (six rotations). Changes in gene frequencies were mitigated when population size was increased to 600 and advanced regeneration was considered in the model. Cutting from below was one method that maintained desirable gene frequencies around 0.5. Once desirable gene frequencies approach zero, the prototype demonstrates that larger tree diameters cannot be attained by merely extending rotation ages and/or skipping cutting cycles.

Keywords: Quercus rubra L., genetic diversity, natural regeneration, simulation.

INTRODUCTION

A joint effort between U.S.F.S. Eastern Region (Region 9) and N.C. Experiment Station was initiated in 1989 to address the need to understand genetic changes in naturally regenerated forest tree populations, with particular emphasis on northern red oak. This need arises from the fact that over 75 percent of harvested forest lands in Region 9 are naturally regenerated (Murphy and Kang, 1989).

Historically, silviculturists and forest managers have influenced genetic properties of naturally regenerated populations without much input from geneticists. A common precept among geneticists has been avoidance of dysgenic practices such as high grading and diameter limit cutting (Synder 1972).

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Defining an undesirable/acceptable level of loss/gain has proven to be frustrating. Limited efforts have been made to determine the changes in genetic quality of stands due to silvicultural treatments, and have generated conflicting results. Wilusz and Giertych (1974) and Yazdani et al. (1985) concluded that certain silvicultural practices significantly influenced the genetic quality of stands, while Neale (1985) and Pitcher (1982) suggested otherwise. Such conflict may be attributed to three causes: (1) local environmental conditions among experiments, (2) varying experimental designs, and (3) statistical significance was the only means of assigning the importance of the observed values.

This inability to clearly define dysgenic practices does not imply that we lack useful genetic principles and/or techniques. These difficulties primarily arise from the limited field information, which is necessary to transform currently available genetic principles into management prescriptions. Communication among researchers and land managers can be enhanced through the use of educational software and decision models.

NATGEN was developed as a visual training tool to provide an appreciation of the potential genetic changes in Lake States' northern red oak following vegetation manipulations. The attractiveness of NATGEN is that the user has fingertip access to information on genetics, regeneration, and growth and can readily "see" the results of specific management practices on a tree population via the computer screen, rather than waiting several years to see the effects of their actions on the ground.

In order for non-geneticists to benefit from this educational software, the model was developed from a silviculturist's point of view. Given what is known in the literature about the genetics of hardwoods, the trait of interest to execute prescriptions and monitor genetic response is tree diameter at breast height (DBH), as compared to modeling height growth.

NATGEN aids the user in evaluating the consequences of silvicultural prescriptions such as high-grading, thinning from below, or thinning from above, by diameter classes. Additional development is on-going to incorporate individual-tree selection into the model.

METHODS

NATGEN was developed using Borland C++ version 2.0. Version 1.0 models a second-growth stand of northern red oak. The first prototype had a population size of 100 trees. The maximum number of trees for the full model is 600, at 20-foot spacing, on six acres. Each individual phenotype is made up of three components: genotype, environmental deviate and site value. No genotype-by-environment interaction term is considered.

The genetic component is stochastically determined using a random number subroutine, characterized by an additive gene model, with one quantitative trait with five loci, and two genes (A and a) at each locus (Mahalovich 1990). Default gene frequencies are set to 0.5 ; however, the user has the flexibility of altering these frequencies. Genetic values for the following

gene combinations at a locus are: AA=2, Aa=1, and aa=0. Individual genotypes are the sums of the five-locus genotypic values.

The user can either choose a default individual tree heritability of 0.2, an average value for tree diameter, or pick another value between 0 and 1. Thereafter, heritabilities change relative to the silvicultural prescriptions selected and the number of individuals following regeneration. Procedures for determining total genetic, phenotypic, and environmental variance follow Mahalovich (1990).

The site value component, is unique, in that some form of "mapping" individuals to the computer screen was needed to carry out management objectives and silvicultural prescriptions from a two-dimensional bird's eye view. Site values can be considered as a "fixed" environmental component of the phenotype.

Users may choose one of two environmental sites. The extreme site values range from 0.0 to 2.0 (no units, variance = 0.15), with a pattern resembling a mixed terrain contour map. The more uniform site values range from 0.1 to 1.0 (no units, variance = 0.04), with a pattern resembling a gentle slope. Site values were selected to serve the purpose of mapping individuals to the screen, but so as not to "wash out" heritability. In other words, an initial heritability with the site values considered as part of the total phenotypic variance had to be within 1% of an initial heritability without site values.

Phenotypic scores are translated into a normal diameter distribution based on an initial age of 80 years (Gevorkiantz and Scholz 1948 and McGill et al. 1991), characterizing a second-growth stand. Tree diameters, represented by varying circle widths, are displayed on a grid corresponding to their site value location.

Cutting/growing intervals are set at a minimum of 20-year intervals, based on Clark and Watt (1971), who reported both damage in advanced oak reproduction during logging and increased mortality following sudden sun exposure, when entry cycles were too frequent. The maximum number of intervals modeled for N=600 is 200 years (i.e., if a tree at initiation (age 80) remained uncut throughout the simulation, it would be 280 years of age upon exiting the program).

Following harvesting by three-inch diameter classes, the user may choose to regenerate trees by a seed tree method (only uncut, reproductively mature trees are parents for the progeny population) or by considering advanced regeneration (reproductively mature trees before and after harvesting are considered as parents for producing the next progeny population). Reproductive maturity in Lake States' northern red oak is defined as a minimum DBH of 10 inches, and a range of 50 to 120 years of age (Mr. Dick Cutler, USDA Forest Service, Nicolet National Forest, Rhinelander, WI, personal communication). Comparisons of total tree number to total number of reproductively mature trees conveys the concept of effective population size.

Diameter growth projection follows the harvest and regeneration modules. Lake States' TWIGS equations (Miner et al. 1988, Brand 1981) are most

reliable for individuals not exceeding 120 years of age. Diameter growth projection is constrained to a maximum age of 150 years.

Version 1.0 is a prototype educational software package, which is presently being tailored to address research objectives. Such applications require replication to determine an average response to a given silvicultural prescription. Problems arise however, when more than one factor is varied among cutting cycles, which then limits the user to the results of one replication.

RESULTS AND DISCUSSION

NATGEN as an Educational Tool

The large population model, $N = 600$ and five loci, is designed to give each user the flexibility of making consistent prescriptions every cycle or to make changes, after reviewing the diameter distribution and residual basal area per acre. NATGEN is constrained to 10 cycles because of the reliability of correctly projecting diameter growth beyond 120 years of age (Miner et al. 1988, Brand 1981). For genetic considerations, it would be ideal to accurately predict diameter growth beyond 200 years of age.

When $N < 600$, the user may choose among: 1) seed tree regeneration (only reproductively mature trees following tree removal are considered as parents), 2) advanced regeneration (i.e., number of reproductively mature parents and their gene frequencies are determined before tree removal), or 3) skipping regeneration and proceeding to grow the trees for 20 years. Option (2) provides a larger effective population size among parents. The two approaches to natural regeneration combined with the reproductive maturity constraints, conveys the concept of effective population size. Results of repeated diameter limit cutting every cycle are readily apparent by the fourth and fifth cycles, since the user has generally exhausted the number of reproductively mature trees for options (1) and (2) and has to skip regeneration and grow the remaining trees for 20 years.

Reports of total genetic, phenotypic, and diameter means and variances are available after the user elects to stop the simulation. If the scientist user level is selected, reports are provided on the change in desirable gene frequency (A gene), along with measures of heterozygosity at the locus-level.

NATGEN as a Research Tool

An extension of NATGEN was developed to permit five replicate runs of selected silvicultural prescriptions under a management objective of optimizing volume production. Two population sizes ($N=100$ and $N=600$), with one and five loci, respectively, were used to characterize tree diameter. Default values were selected at initiation (heritability = 0.2, with equal gene frequencies per locus, $A = a = 0.5$). Diameter limit cutting (18+ inches) or diameter limit cutting combined with cutting from below (18+ inches and ≤ 9 inches in DBH) were modeled for both population sizes. Regeneration was based on the seed tree method.

For the N=100 one-locus model, natural regeneration followed harvesting at rotation (80 years). Harvesting occurred every 80 years. The desirable gene frequency (A-gene) reached a frequency of 0.1 as early as six rotations under both harvesting regimes for the extreme site type (Figure 1). The A-gene frequency and total genetic variance were unable to recover under diameter limit cutting; however, slightly higher levels of genetic variance were maintained with the combined 18/9-inch harvest regime. Diameter distributions changed over time as the original population was carried for 10 rotations, with fewer trees represented in the larger diameter classes (Figure 2).

For the larger model (N=600 and five loci), natural regeneration could occur every 20-year cycle if $N < 600$ and at least two remaining trees had reached reproductive maturity. Desirable gene frequencies among the five loci ranged from 0.5 (± 0.01) at initiation, to 0.48 (± 0.02) at 280 years for both site types. Gene frequencies at 280 years are higher than the one-locus model at three rotations due to an increase in population size (Figure 1). Amounts of genetic variance differed between sites for diameter limit cutting (18+"E, 18+"U) (Figure 3). Total genetic variance was more stable when diameter limit cutting occurred at cycles 1, 5, and 9 and cutting from below occurred at cycles 3 and 7 (18ALT9"E and 18ALT9"U, Figure 3).

Changes in diameter distributions for the extreme site (Figure 4) yielded more trees in the lower diameter classes over time, for both harvesting regimes. Diameter limit cutting resulted in a larger number of total trees, with trees represented in the lower diameter classes (Figure 4). Fewer numbers occurred in the combination harvesting regime (18ALT9"E) at 280 years, since the number of reproductively mature trees was the limiting factor in natural regeneration.

For the uniform site, the number of reproductively mature trees was not a limiting factor, hence, there were equal numbers of trees at 280 years for both harvesting regimes (Figure 5). Changes in diameter distributions over time were noted only in the proportion of trees represented in each diameter class by harvesting regime, but not in total numbers.

Only one factor could vary among replicate runs, in order to get an average response. In some cycles however, when harvesting was scheduled to occur, residual basal area per acre was below 80 square feet. In practice, no harvesting would have occurred so as to maintain desirable stocking levels (McGill et al. 1991), and tree removal would have been delayed until residual basal area per acre exceeded 80 square feet.

CONCLUSIONS

There are genetic consequences for each management objective and combination of silvicultural prescriptions on tree populations. Results of these simulation trials demonstrated both a reduction in genetic variance, desirable gene frequency, and changing diameter distributions, with fewer individuals in the larger diameter classes in later years. The full model

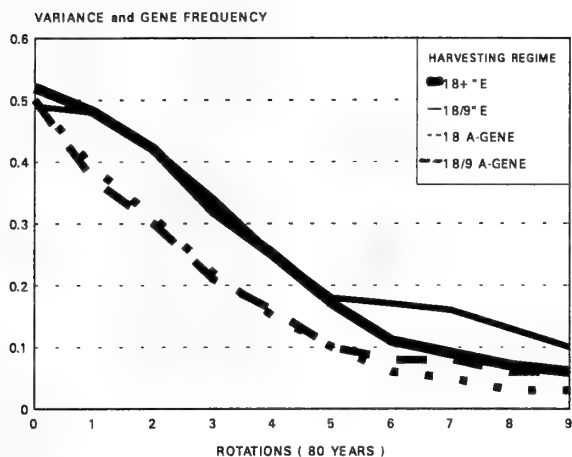


Figure 1. Total genetic variance and A-gene frequency following seed tree regeneration for one locus and N= 100 trees.

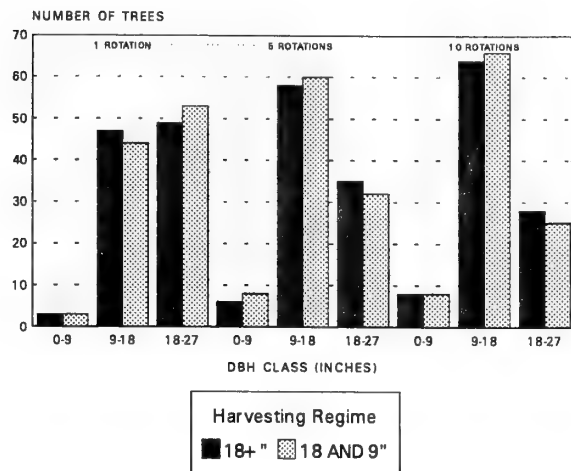


Figure 2. Diameter distributions by harvesting regime, at one, five and 10 rotations, for N= 100.

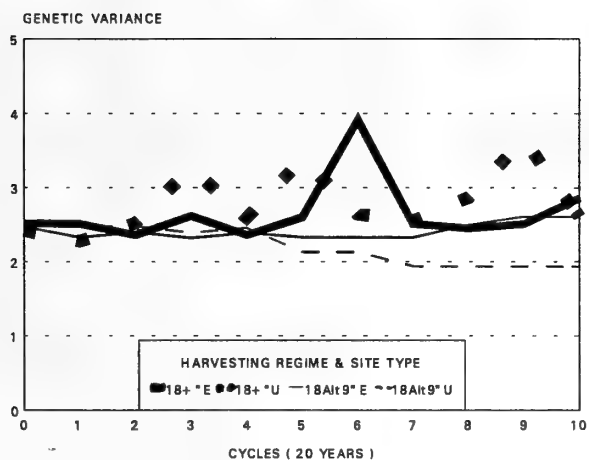


Figure 3. Total genetic variance following seed tree regeneration for five loci and N= 600 trees.

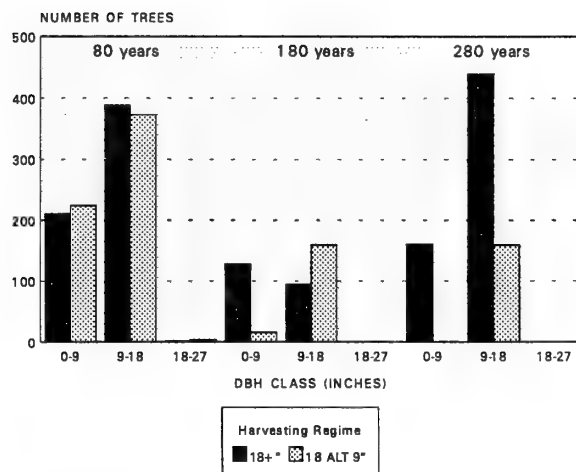


Figure 4. Extreme site (E) diameter distributions by harvesting regime, at cycles 0, 5 and 10, for N = 600.

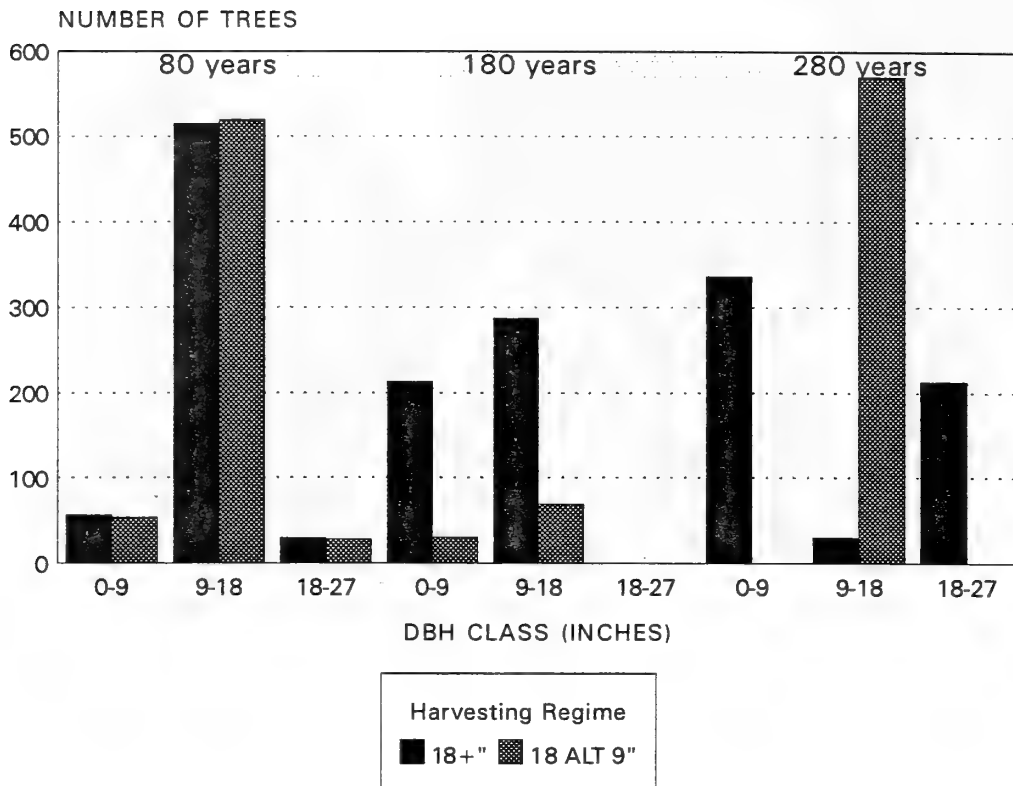


Figure 5. Uniform site (U) diameter distributions by harvesting regime, at cycles 0, 5 and 10, for N = 600.

demonstrates that with declining gene frequencies and fluctuations in the number of reproductively mature parents, large diameter trees cannot be achieved merely by skipping cuttings cycles or by extending rotation ages in an uneven-aged system. The results reported would be more conservative if mortality functions had been included or if natural regeneration patch sizes had been limited to no smaller than one-fifth of an acre (Lorimer 1983). Selection of advanced regeneration would offset the decline in gene frequencies and total genetic variance, since the effective population would be expected to be higher than with the seed tree method.

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STUDIES ON GENETIC DIVERSITY IN EUROPEAN OAK POPULATIONS

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Abstract. The possession of genetic variation is an indispensable precondition for the ability of forest tree populations to survive spatial and temporal variation of the environment. Therefore, genetic variation is the basis of any evolutionary development. Genetic inventories give an idea of the amount of genetic variation in a forest tree population. In European oaks, with the exception of some recent studies in juvenile populations of pendunculate oak (*Quercus robur*) and sessile oak (*Quercus petraea*), employing biochemical genetic marker systems, such inventories do not yet exist. The results of the present study using biochemical markers on different European populations of sessile oak revealed relatively small genetic variation within individuals in terms of heterozygosity. In contrast, the variation within populations is large. Genetic differentiation among populations of each species is relatively small. In contrast to earlier results, which yielded smaller values for pendunculate oak as compared to sessile oak, the present results indicate the opposite trend. As can be expected, mixtures of population samples from different locations are less differentiated than samples from single locations. The genetic information of pendunculate oak and sessile oak is quite similar. So far, no species-specific alleles have been found. However, allele frequencies can vary between species. In particular, genetic distances are found to be greater between populations of the two different species than between populations of the same species. Oaks are carrier tree species of important forest ecosystems. Compared to other species, oaks are long-lived and thrive in a wide range of ecological site conditions. These peculiarities should require very large intrapopulation variation and in fact, such variation was observed in all previous preliminary studies. However, many alleles are rare in all populations in which they occur. As a consequence, further intensive studies on genetic structures with special consideration of appropriate measures for the conservation of genetic resources are required.

Keywords: *Quercus*, genetic variation, genetic resources.

INTRODUCTION

Oaks belong to the major deciduous tree species in Europe. Two species, *i.e.* *Quercus robur* (pedunculate oak) and *Quercus petraea* (sessile oak) are quantitatively predominating in most parts of Europe. They are carrier species of complex, economically as well as ecologically important forest ecosystems. They range from the

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fertile plains of the lowlands to the submontane or even montane regions. Oaks are relatively long-lived species with forest rotation cycles of 200 and more years. Thus, they are exposed to more heterogeneous environmental conditions than most other predominant tree species and may serve as model organisms in the study of genetic variability and its implications for the survival of tree populations, especially in complex environmental situations.

The objective of the present study is to proceed in the characterization of the genetic variation in pedunculate oak and sessile oak populations and to supplement the genetic inventory of European oak populations initiated by preliminary studies of MÜLLER-STARCK and ZIEHE (1991), KREMER *et al.* (1991), and MÜLLER-STARCK *et al.* (in press). Additionally, it should contribute to the knowledge of the natural variability of forest ecosystems in general. Data on patterns of genetic variation will lead to a better understanding of principles of adaptation and survival of long-lived tree species. Such data is needed to develop criteria for the choice of reproductive material, for silvicultural treatment as well as for the conservation of genetic resources.

MATERIAL AND METHODS

Nine stands of sessile oak (*Quercus petraea* LIEBL.) from Scotland, France, Danmark and Deutschland were studied (Figure 1).

The samples represent presumably indigenous populations of sessile oak and also include marginal provenances of this species. The sample size was 100 trees per population. In addition, first results of an unpublished study of HERZOG and KRABEL on pedunculate oak *Quercus robur* are discussed in the context of the present study.

Buds or young leaves were sampled and immediately frozen in liquid nitrogen before storage at -80° Celsius. In the present study the sample size was 100 per population. This means a probability of 95 per cent to detect alleles with a frequency of at least $\alpha=5.99$.

Isoenzyme analysis was modified following MÜLLER-STARCK and ZIEHE (1991). The buds were thawed and homogenized in a 0.08/0.02 mol/l TRIS/HCl buffer at pH 7.3. To inhibit phenols and tannins, 2 to 5 %[w/v] polyvinylpyrrolidone, 10 to 130 mmol/l mercaptoethanol, 3 mmol/l ethylenediaminetetraacetic acid (EDTA) as well as 3 to 6 mmol/l dithiothreitol were added. The resulting slurry was absorbed onto filter paper wicks and loaded onto gel slabs. Horizontal starch gel electrophoresis was performed using a starch concentration of 11.5%[w/v]. The bridge distance was 12 cm with a voltage distribution of 20 to 30 V/cm.

Six isoenzyme systems (table 1) representing seven polymorphic gene loci, were identified by MÜLLER-STARCK and HATTEMER (1990) as well as HERZOG (unpublished data) as genetic markers.

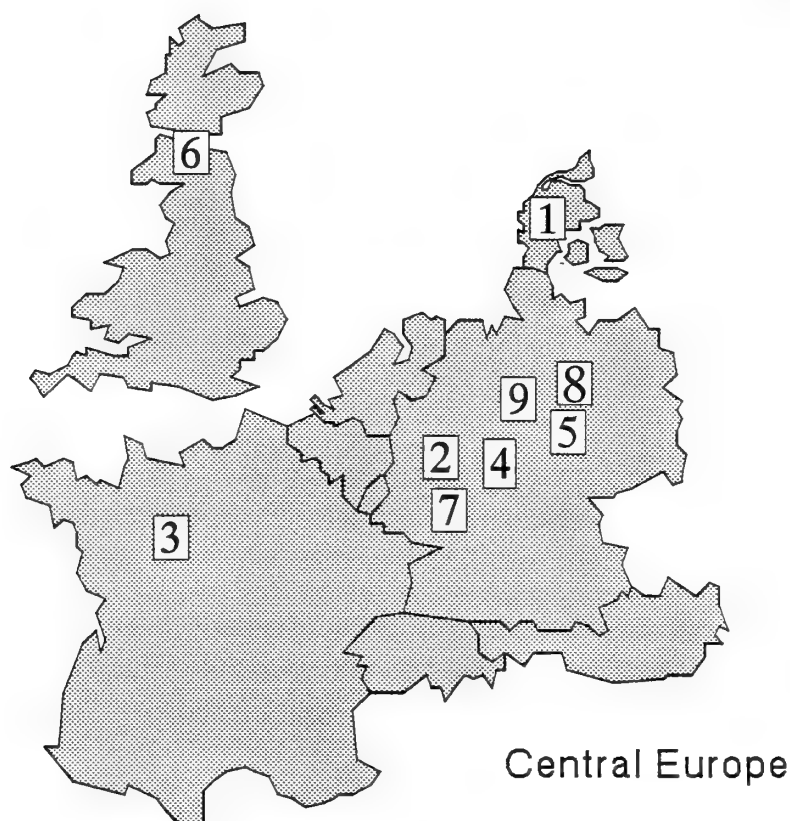


Figure 1. Schematic map of Central Europe and the populations studied (for identification of the single populations see Table 2).

They were studied using different electrode and gel buffer systems (table 1), modified following MÜLLER-STARCK and ZIEHE (1991). Solutions used for enzyme staining were modified following CHELIAK and PITEL (1984).

RESULTS AND DISCUSSION

Variation within populations

One commonly used measure for genetic variation of individuals and populations is the degree of heterozygosity (table 2). Its estimation relies on genotypic rather than on allelic frequencies; the amount of heterozygosity attainable in a population ("actual amount of heterozygosity", H_A) depends on the actual allele frequencies.

The present study revealed an average (arithmetic mean) actual heterozygosity of $H_A=0.229$ over 9 populations of *Quercus petraea* (table 2).

The highest value was found for Scotland (Dymock), the lowest value for one population from the north of Deutschland (Lüß). The average lies near to the upper bounds

Table 1. *Enzyme systems and buffers used in the present study.*

Enzyme system and E.C. code	Electrode buffer and Gel buffer
Shikimate dehydrogenase E.C. 1.1.1.25	0.14/0.05 mol/l TRIS/citric acid, pH 6.5 0.04/0.014 mol/l TRIS/citric acid, pH 6.6
Isocitrate dehydrogenase E.C. 1.1.1.42	0.14/0.04 mol/l TRIS/citric acid, pH 7.8 0.04/0.011 mol/l TRIS/citric acid, pH 7.8
6-phosphogluconate dehydrogenase E.C. 1.1.1.44	0.14/0.04 mol/l TRIS/citric acid, pH 7.8 0.04/0.011 mol/l TRIS/citric acid, pH 7.8
Glutamate dehydrogenase E.C. 1.4.1.2	0.06/0.30 mol/l NaOH/boric acid, pH 8.0 0.07/0.02 mol/l TRIS/HCl, pH 8.7
Phosphoglucumutase E.C. 2.7.5.1	0.14/0.05 mol/l TRIS/citric acid, pH 6.5 0.04/0.014 mol/l TRIS/citric acid, pH 6.6
Phosphoglucose isomerase E.C. 5.3.1.9	0.05/0.19 mol/l LiOH/boric acid, pH 8.1 0.05/0.01 mol/l TRIS/citric acid, pH 8.1

of the spectrum given by previous studies for this species: in previous studies, the average heterozygosity at the species level was estimated to be $H_A=0.213$ for *Quercus robur* and 0.219 resp. 0.229 for *Quercus petraea* by MÜLLER-STARCK and ZIEHE (1991) and MÜLLER-STARCK *et al.* (in press). HERZOG and KRABEL (unpublished data) found an average of $H_A=0.253$ for *Quercus robur*.

Deviations from Hardy-Weinberg proportions were observed at several loci, but in most cases expected genotype frequencies are less than 5, which may erroneously suggest rejection of the hypothesis using the χ^2 -test. However, provided the deviations were truly significant, they may suggest an influence of selection or the mating system, but the present data base does not allow further conclusions in this context.

Diversity is measured using the gene pool diversity (v , GREGORIUS 1978, 1987) and the total population differentiation (δ_T^* , GREGORIUS 1987; table 2). Especially the calculation of v makes the data comparable to other studies. Whereas the present study revealed a total population differentiation δ_T^* ranging between 0.210 and 0.322, the gene pool diversity v was found to range between $v=1.26$ and $v=1.47$. MÜLLER-STARCK and ZIEHE (1991) as well as MÜLLER-STARCK *et al.* (in press) calculated gene pool diversities between $v=1.29$ und $v=1.49$ for *Quercus petraea*. For *Quercus robur* the respective values were found to range between $v=1.33$ and $v=1.41$ (MÜLLER-STARCK and ZIEHE 1991, MÜLLER-STARCK *et al.* in press) resp. between $v=1.35$ and $v=1.47$ (HERZOG and KRABEL unpublished data).

Table 2. Genetic parameters for 9 sessile oak (*Quercus petraea*) populations: heterozygosity (H_A), gene pool diversity (v , GREGORIUS 1978, 1987), population differentiation (δ_T^* , GREGORIUS 1987), and subpopulation differentiation (D_j , GREGORIUS und ROBERDS 1986).

Origin	H_A	v	δ_T^*	D_j
(1) Danmark (Horbylunde)	0,227	1,47	0,322	0,103
(2) Deutschland (Bad Münstereifel)	0,211	1,34	0,257	0,059
(3) France (Youille Saint Hilaire)	0,217	1,33	0,252	0,052
(4) Deutschland (Wolfgang)	0,253	1,36	0,266	0,048
(5) Deutschland (Lüß)	0,191	1,26	0,210	0,049
(6) Scotland (Dymock)	0,261	1,30	0,230	0,044
(7) Deutschland (Johanniskreuz)	0,233	1,34	0,254	0,045
(8) Deutschland (Göhrde)	0,224	1,33	0,250	0,074
(9) Deutschland (Seelzerthurm)	0,246	1,35	0,262	0,078

Thus, the present data correspond well to previous studies on oak populations. However, the values are high compared to the results of studies on other plant species. HAMRICK and GODT (1990) reanalyzed more than 600 studies and found an average "effective number of alleles" of 1.24. This measure is comparable to the diversity v what means a relatively high diversity of oaks. This may result from the above mentioned spatial and temporal heterogeneity of the environments.

Differentiation between populations

In the present study, the genetic distances d_0 (GREGORIUS 1974, 1984) as well as the gene pool subpopulation differentiation D_j (see Table 2) and δ (GREGORIUS and ROBERDS 1986, GREGORIUS 1987; Table 3 and 4) were applied.

Table 3. Pairwise genetic (gene pool) distances d_o between populations of sessile oak (*Quercus petraea*)

	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
(1)	—								
(2)	0,088	—							
(3)	0,088	0,071	—						
(4)	0,114	0,079	0,039	—					
(5)	0,114	0,075	0,042	0,053	—				
(6)	0,098	0,054	0,053	0,045	0,041	—			
(7)	0,108	0,081	0,061	0,056	0,054	0,061	—		
(8)	0,150	0,106	0,077	0,064	0,078	0,087	0,070	—	
(9)	0,157	0,106	0,090	0,073	0,071	0,071	0,077	0,061	—

Genetic distances d_o s of, say, 0.1 and more provide evidence for a substantial genetic differentiation. However, the genetic distances are poorly correlated to the geographical distances. This means that we also should not expect a good correlation between genetic differentiation and geographic distance of the sites.

The subpopulation differentiation was found to range between 0.044 (Dymock, Scotland) and 0.103 (Horbylunde, Danmark). Thus, the subpopulation differentiation δ (GREGORIUS 1987; Table 4) is equalling 0.061; the corresponding value was found to be 0.055 in *Quercus robur* and 0.085 in *Quercus petraea* (MÜLLER-STARCK and ZIEHE 1991). Another study on *Quercus robur* revealed $\delta=0.091$ (HERZOG and KRABEL unpublished data).

Table 4. Subpopulation differentiation δ (GREGORIUS und ROBERDS 1986): comparison of the studies to date.

Species (author)	δ
<i>Quercus petraea</i> (this study)	$\delta=0,061$
<i>Quercus petraea</i> (MÜLLER-STARCK and ZIEHE 1991)	$\delta=0,085$
<i>Quercus robur</i> (MÜLLER-STARCK and ZIEHE 1991)	$\delta=0,055$
<i>Quercus robur</i> (HERZOG and KRABEL unpublished data)	$\delta=0,091$

These results provide evidence that the adult stands of pedunculate oak are

more differentiated than the juvenile populations of pedunculate and sessile oak. Moreover, the present data show a reduced δ for sessile oak compared to the studies of MÜLLER-STARCK and ZIEHE (1991) as well as MÜLLER-STRACK *et al.* (in press). However, this may be at least partially caused by differences in the genetic structures dependent on age or by the restricted number of populations studied by HERZOG and KRABEL (unpublished data) (2 populations) or the latter authors (5 populations). The study of KREMER (1991) found juvenile populations of sessile oak especially in France to be significantly less differentiated ($G_{ST}=0.017$). This may be partially caused by methodological differences (δ values normally exceed the corresponding G_{ST} values), but nevertheless it can be concluded that sessile oak in France may be less differentiated than in other parts of Europe studied to date. In general, the genetic differentiation of oak populations is of the same magnitude as observed for other deciduous tree species (see also MÜLLER-STARCK 1991).

Another problem to be discussed is that of rare alleles. The common differentiation measures normally underestimate the influence of alleles occurring in low frequencies, say, less than 10 per cent. For example, we can consider the *PGM-A* gene locus (Table 5, Figure 2).

One allele (*PGM-A₄*) is predominating and shows a high frequency of more than 80 per cent with one exception (Hörbylunde). The other rare alleles are heterogeneously distributed what provides evidence for a locally acting selection against different rare alleles, whereas the common allele may be optimized under the present environmental conditions in general. We have to keep in mind that this differentiation pattern caused by rare alleles does not cogently correspond to that revealed by application of the common differentiation measures.

Consequences for conservation of genetic resources

Genetic variation is an important prerequisite for the ability of forest tree populations to survive spatial and temporal variation of environmental conditions. Therefore, genetic variation is the basis of any evolutionary development. Consequently, provisions on gene conservation require an inventory of genetic variation in as many populations of a species as possible. The present study should contribute to this inventory.

The present results reveal a relatively small genetic variation within individuals in terms of small actual heterozygosities. In contrast to that, intrapopulation variation is extraordinarily large.

In general, genetic differentiation among populations of each species is relatively small. In contrast to earlier results which suggest smaller values for pedunculate oak as compared to sessile oak, the present results indicate the opposite trend. As can be expected, mixtures of population samples from different locations are less differentiated than samples from single locations.

The gene pools of pedunculate oak and sessile oak are very similar: There are no species specific alleles so far although allele frequencies can vary between species.

Table 5. Differentiation between sesile oak populations (*Quercus petraea*) due to the occurrence of rare alleles using the gene locus *PGM-A* as an example (see also Figure 2)

	PGM-A ₁	PGM-A ₂	PGM-A ₃	PGM-A ₄	PGM-A ₅	PGM-A ₆
Horbylunde	0.000	0.365	0.020	0.565	0.050	0.000
Bad Münst.	0.000	0.070	0.025	0.885	0.020	0.000
Y.-St.-Hilaire	0.000	0.005	0.005	0.935	0.035	0.020
Wolfgang	0.005	0.000	0.015	0.925	0.030	0.025
Lüß	0.000	0.000	0.000	0.985	0.015	0.000
Dymock	0.000	0.035	0.000	0.945	0.020	0.000
Johanniskreuz	0.000	0.105	0.025	0.840	0.025	0.005
Göhrde	0.000	0.030	0.000	0.910	0.055	0.005
Seelzerthurm	0.000	0.020	0.000	0.975	0.005	0.000

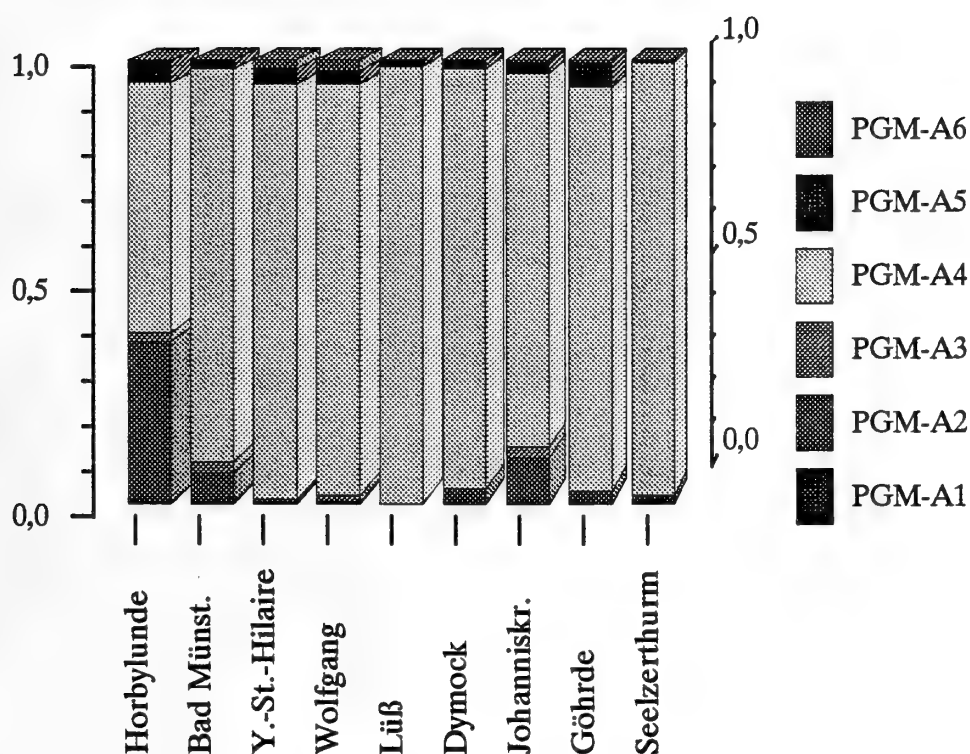


Figure 2. Differentiation between sesile oak populations (*Quercus petraea*) due to the occurrence of rare alleles using the gene locus *PGM-A* as an example (see also Table 5)

Consequently, genetic distances are greater between populations of the two different species as compared to populations within each species.

As above mentioned, oaks are carrier tree species of significant forest ecosystems. Compared to other tree species, oaks are extremely long-lived and cover a wide range of ecologically different forest sites. Genetic resources of oaks are endangered not only by the loss of living spaces such as the natural fertile plains but also by the impact of air pollution for several decades and maybe even by long-term climatic changes. Moreover, silvicultural customs, especially the limitation of seed sources may also contribute to the loss of genetic resources. In addition, since several years we can observe an increasing impact of air pollutants even on broad-leaved trees such as oaks and beech. The results presented herein suggest that forest tree breeding and silviculture of sessile oak and pedunculate oak need to take into account large genetic multiplicities. Genetic heterogeneity should correspond to the tremendous environmental heterogeneity to which long-lived oak populations are exposed (see also MÜLLER-STARCK *et al.* in press). It appears that large genetic variation has to be incorporated in productive populations in order to maintain the potential of these populations to adapt to and to survive in complex environmental situations.

It seems not probable that we can find single stands representing the whole or nearly the whole genetic variation of the species. This would call for a management which is focussed on the *in situ* maintainance of numerous and sufficiently large, locally adapted stands. First rough estimations recommend minimum sizes of oak stands serving for gene conservation purposes of 30 to 50 hectares (HERZOG and MÜLLER-STARCK 1993). However, the present results provide good evidence that genetic conservation of oaks should be possible by means of a regular silvicultural management.

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SESSION 5

Gene Mapping

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and D.B. Neale^{1/}

Abstract. We are evaluating several approaches to identify and map expressed genes with known functions to create a transcriptional, or gene, map of the loblolly pine (Pinus taeda L.) genome. Several approaches to identify expressed genes are: 1) sequencing random complementary DNA (cDNA) from loblolly pine, 2) obtaining gene probes from other species of Pinaceae, 3) cloning gene fragments using the polymerase chain reaction, and 4) constructing cDNA libraries that represent specific classes of expressed genes. cDNA clones representing expressed genes are used as probes to detect and map RFLP loci. The putative identities of nine mapped cDNA's for loblolly pine have been determined, eight RFLP loci have been mapped using probes from P. sylvestris, and a segment of a phytochrome gene has been cloned using PCR primers designed from Arabidopsis thaliana phytochrome coding sequences. The results of our pilot studies will help determine the future emphases of our gene mapping efforts.

Keywords: Pinus taeda L., genetic map, expressed genes

INTRODUCTION

Integrating information on DNA sequence, chromosomal location, and expression pattern of transcribed genes is an important goal of genetic mapping research. A transcriptional map, a genetic map that reveals the chromosomal positions of expressed genes, is being constructed for the loblolly pine (Pinus taeda L.) genome. The map will: 1) further our understanding of conifer genome structure and allow comparison to other plant genomes, 2) facilitate the manipulation of genes, 3) provide a set of DNA probes for assessing genetic diversity and tree health in forest populations, and 4) organize a large body of related genetic information through electronic databases.

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Researchers within large-scale mapping projects, such as the human genome project, are identifying expressed genes using many novel approaches. Such approaches include computational methods, exon trapping, and recombination-based methods (Hochgeschwender, 1992). Large scale sequencing and mapping is also being applied to Arabidopsis thaliana, and researchers are attempting to identify and map over 20,000 expressed sequences using PCR-based approaches. Conifer genome research is presently being advanced on a comparatively small scale. Less than 30 conifer DNA sequences, representing different nuclear-encoded genes, have been submitted to Genbank, the DNA sequence database, and the first genetic maps have only recently been completed.

Genetic maps are being constructed in conifers using several different marker types including isozymes (Conkle 1981), restriction fragment length polymorphisms (RFLP's) (Neale and Williams 1991), and random amplified polymorphic DNA (RAPD) (Grattapaglia et al. 1992, Tulsierium et al. 1992). We have constructed two RFLP maps based on three generation outbred pedigrees using complementary DNA (cDNA) probes. The cDNA library was constructed by random priming of messenger RNA (mRNA) from two-week-old loblolly pine seedlings (Devey et al. 1991). Gene mapping in loblolly pine with cDNA probes is generally easy, because loblolly pine exhibits enormous genetic variation, and variation is easily detectable with RFLP's. The "base" map consists of 90 loci and 20 linkage groups, while the wood specific gravity (WSG) map, constructed for detecting genetic markers linked to loci determining wood specific gravity, consists of 175 loci and 23 linkage groups (Groover et al. 1993). These maps will be further developed and eventually merged to create a consensus map for loblolly pine with linkage groups representing the 12 chromosomes.

Conifer genomes are unusually large and complex compared with genomes of most commonly studied plants; consequently, constructing a transcriptional map in loblolly pine using RFLP's involves special challenges. Southern blots using individual cDNA probes typically contain many bands (Devey et al. 1991). We routinely map several loci using a single cDNA probe, but some loci may not be segregating in our pedigrees, or banding patterns may be too complex to interpret. Therefore, cDNA probes do not usually correspond one-to-one with genetic loci. Multiple bands on Southern blots may represent gene family members, pseudogenes, or other uncharacterized products of gene amplification. The relative complexity of banding patterns between pines and commonly studied crop species may reflect differences in the molecular mechanisms by which conifer and angiosperm genomes have evolved, and it is likely that gene family complexity impacts the expression of individual family members within pines (Kinlaw and Gerttula 1993).

Our initial goal is to identify and map 500 expressed loblolly pine genes using cDNA probes and RFLP maps. The approaches to identify genes are: 1) sequencing loblolly pine cDNA's that were previously mapped but were otherwise uncharacterized, 2) utilizing gene probes from other species of Pinaceae 3) cloning gene probes by the polymerase chain reaction (PCR), and 4) constructing cDNA libraries that represent specific classes of genes. We will briefly describe each of these methods and present some preliminary data.

METHODS AND PRELIMINARY RESULTS

Sequencing and Mapping cDNA's

This approach involves four basic steps: 1) isolating random cDNA's from a complex mRNA population, 2) mapping loci revealed by the cDNA probes, 3) sequencing cDNA's that correspond to mapped loci, and 4) performing database searches for sequence similarity to determine cDNA identity. For the initial study, we selected two-hundred loblolly pine seedling cDNA clones, most for which one or more loci have been placed on RFLP maps. Approximately 200 nucleotides from both ends of the clones are now being determined by Chris Baysdorfer's group (Keith et al. 1993) for gene identification by similarity searching with the Genbank database using the Fastdb algorithm (Intelligenetics, Mountain View, CA).

In a preliminary test, sequences for 35 of the cDNA's described above were submitted to Genbank. Ten (28%) resulted in matches to known genes at a 60% or greater identity criteria (Table 1). We consider 60% identity to indicate significant sequence relationships, because conifers are evolutionarily divergent from all other organisms represented in Genbank. Previous DNA sequence comparisons of conifer alcohol dehydrogenase and light harvesting complex sequences with those of other plants were used to establish the 60% identity criteria for sequence identification (Kinlaw et al. 1990, Jansson 1992).

The frequency of clones in cDNA libraries is roughly proportional to messenger RNA abundance. Consequently, the results shown in Table 1 reflect redundancy for highly abundant photosynthetic genes such as those encoding light harvesting complexes and the small subunit of *rbcS*. Redundancy for such genes was expected, since no attempt was made to minimize it, and the messenger RNA source seedlings for the loblolly pine cDNA library were photosynthetically active. Future studies, described below, will be devoted to generating cDNA libraries enriched for specific and depleted for abundant messages. These pilot experiments provide evidence that sequencing and database similarity comparisons of unknown loblolly cDNA's with even very distantly-related organisms is a powerful strategy for gene identification that makes efficient use of available information.

Table 1. Results of partial sequencing and subsequent Genbank similarity searches for 35 loblolly pine cDNA's.

Loblolly Pine Clone	Linkage Group ^a		Putative Gene	Gene Source
	Base Map	WSG Map		
pPt1IFG2253	-	-	Aldolase	Spinach
pPt1IFG1599	5	16	ATPase	Spinach
pPt1IFG1934	1	1	<i>Lhcb1</i> *2 ^b	Scots pine
pPt1IFG1584	-	16	Deoxychalcone synthase	Soybean
pPt1IFG2022	2	10	Glutamine synthetase	French bean
pPt1IFG2357	2	-	Glutamine synthetase	Lettuce
pPt1IFG2166	-	23	Pyruvate dehydrogenase	Mouse
pPt1IFG2025	1	-	<i>rbcS</i> ^c	Japanese black pine
pPtIFG1635	1	-	<i>rbcS</i>	Japanese black pine
pPtIFG669	4	14	<i>psbS</i> ^d	Spinach

^aLinkage group numbers, assigned arbitrarily, will be reconciled.

^bLight harvesting complex (for *cab* nomenclature, see Jansson 1992)

^cRibulose biphosphate carboxylase small subunit

^dPhotosystem II 22 kDa polypeptide

Mapping Gene Probes from Other Pines

cDNA and genomic clones from other *Pinus* species and other Pinaceae genera can be used as heterologous probes for detecting and mapping similar genes in loblolly pine. We have shown that cDNA probes from loblolly pine can be used as probes for other members of Pinaceae (Ahuja et al. In prep.). Loblolly pine cDNA probes are currently being used to construct RFLP maps for Monterey pine, Scots pine, Douglas-fir, and Norway spruce. These studies provide ample evidence that probes from other pine species will cross-hybridize to loblolly pine DNA.

We have mapped several loci using cDNA probes from other pines, indicating that hybridizing loblolly pine Southern blots with heterologous probes is a useful strategy for gene mapping in pines (Table 2). Interestingly, the LHC probe S13 from *P. sylvestris* mapped directly to the same position as the locus 1934a, detected by a loblolly pine seedling random cDNA probe. Subsequent sequencing of the cDNA identified it as a loblolly pine LHC gene. The success of this strategy depends upon the amount of sequence homology between loblolly pine and other conifers and on the ability to detect variation in mapping populations with heterologous probes.

We encourage researchers involved in cloning conifer genes to send cDNA or genomic clones to us, and we will attempt to

determine the chromosomal position of corresponding genes in the loblolly pine genome. For example, we are attempting to map genes involved in lignin biosynthesis (Whetten and Sederoff 1992) and plant defense. The results of experiments conducted with contributed probes will be made available in the forest tree genome database, Dendrome (Sherman and Neale 1993).

Table 2. *P. sylvestris* cDNA probes positioned on *P. taeda* WSG RFLP linkage map.

Probe I.D.	<i>P. taeda</i> Locus	Linkage Group	Probe Source
Lhcb1A ^a	S1a	2	Jansson, 1992
Lhcb2	S6a	10	"
Lhcb1B	S13a	3	"
Lhca3	S43a	16	"
Lhca3	S43d	16	"
Lhca3	S43b	30	"
Lhca3	S43c	30	"
CuZn-SOD ^b	Ps3a	10	Karpinski, 1992

^aLight harvesting complex

^bCuZn-superoxide dismutase

PCR Cloning Loblolly Pine Genes

While many angiosperm genes have been cloned and sequenced, relatively few gymnosperm genes have been isolated and characterized. We wish to utilize DNA and protein sequence information available in Genbank and other sequence databases to identify and then map loblolly pine genes with important functions. Therefore, we are assessing the potential of PCR-based cloning strategies for identifying transcribed sequences with known functions in loblolly pine.

Queries are made to Genbank or other sequence databases to determine whether or not a gene of interest is represented. Sequences from other plants are then compared to identify conserved domains using multiple alignment software. Degenerate PCR primers are subsequently designed to the conserved regions. Primers are annealed to loblolly pine mRNA, cDNA, or genomic DNA templates, and corresponding gene fragments are amplified. Various PCR strategies involving different template-primer combinations could be used, including:

- 1) two degenerate primers representing highly conserved regions, with a genomic DNA template;
- 2) one degenerate and one oligo(dT) primer, with a cDNA or RNA template; or

- 3) one degenerate primer to a highly conserved region and one random primer, with a genomic DNA template.

Special considerations will influence the effectiveness of each strategy, such as the presence of introns, quality of messenger RNA or cDNA, or amplification of artifacts. Success will depend on the amount and patterns of sequence conservation of loblolly pine genes to other plant genes of interest, effective design of functional PCR primers, and ability to match appropriate PCR templates with primers. We hope that these strategies will allow sequence information from angiosperms and distantly-related gymnosperms to accelerate loblolly pine gene mapping.

Some preliminary results in Douglas-fir for a gene encoding phytochrome highlight one successful example of a PCR-based cloning strategy that we believe will also be successful in loblolly pine. PCR primers were designed to two highly conserved domains of phytochrome polypeptides of Arabidopsis thaliana (Figure 1).

Figure 1. Arabidopsis thaliana phytochrome polypeptide fragments and consensus sequence used for designing degenerate PCR primers (underlined) for Douglas-fir phytochrome gene PCR cloning (Modified from Sharrock and Quail 1989).

	400
phy A...	CGSTLRAPHSCHLQYMANMDSIASLVMAVVVNEEDGEGDAPDATTQPQKR
phy B...	VGSTLRAPHGCHSQYMANMGSIASLAMAVIINGNEDDGSNVASG...RSS
phy C...	SGSTLRAPHGCHAQYMSNMGSVASLVMSVTINGSDSDENMRDL....QTG
Cons	GSTLRAPH CH OYM NM S ASL M V N
	450
phy A	KRLWGLVVCHNTTPRFVFPFLRYACEFLAQVFAIHVNKEVELDNQMVEKN
phy B	MRLWGLVVCHHTSSRCIPFPLRYACEFLMQAFGLQLNMELQLALQMSEKR
phy C	RHLWGLVVCHHASPRFVFPFLRYACEFLTQVFGVQINKEAESAVLLKEKR
Cons	LWGLVVCH R PFPLRYACEFL Q F N E EK
	500
phy A	ILRTQTLLCDMLMRDAPLGIVSQSPNIMDLVKCDGAALLYKDKIWKLGTT...
phy B	VLRTQTLLCDMLLRDSPAGIVTQSPSIMDLVKCDGAFLYHGKYYPLGVA...
phy C	ILQTQSVLCDMLFRNAPIGIVTQSPNIMDLVKCDGAALYYRDNLWSLGV...
Cons	L TQ LCDML R P GIV QSP <u>IMDLVKCDGAA</u> Y LG

A PCR reaction was conducted using Douglas-fir genomic DNA as template, resulting in the expected 400 base pair DNA fragment. The fragment was cloned, and the nucleotide sequence determined. The nucleotide sequence of the 400 bp amplification product was searched for similarity with Genbank sequences using Fastdb, and the fragment shared 66% identity with phy A from A. thaliana, confirming it to be a phytochrome gene fragment. The PCR clone was then cross-hybridized to Southern blots containing Douglas-fir and loblolly pine mapping population DNA's. Based on the hybridization pattern, ten or more phytochrome genes appear to exist in the loblolly pine genome, several of which are

polymorphic in our mapping pedigree. These results suggest that this PCR-based approach will be successful for other genes represented in sequence databases.

Constructing Specialized cDNA Libraries

Gene libraries are often generated to represent classes of expressed genes that are tissue-specific, developmentally regulated, or environmentally controlled, and such libraries are a useful gene mapping tool. For example, specific libraries are being used in the human genome project to map genes from several important organs. Methods for generating these libraries include subtractive hybridization, differential screens, and new chromosome specific techniques. In loblolly pine, we plan to make cDNA libraries representing wound-inducible, meristem, leaf, root, and inflorescence-specific classes of genes. Northern blots containing RNA from different tissues, developmental stages, and inducible conditions will be used to confirm cDNA specificity, and further evaluate patterns of gene expression.

DISCUSSION

Preliminary results for the identification and mapping of loblolly pine genes are encouraging. Partial sequencing and Genbank database searches have revealed the putative identities of 10 of 36 (28%) loblolly pine random cDNA clones, a match rate that is similar to the one found in comparable experiments in Zea maize (Keith et al. 1993). A 28 percent match rate represents a viable gene identification strategy, and the rate is a remarkable indicator for gene conservation between conifers and other plants. We plan to continue mapping random cDNA's and will routinely sequence and search for similarity for these clones.

Light harvesting complex and CuZn-superoxide dismutase cDNA's from Scots pine have been successfully used as RFLP probes to loblolly pine DNA. We will continue mapping clones from other species, but this method is slow because the rate at which conifer genes are being cloned is slow. PCR strategies, such as the example with phytochrome, have great potential, but must be further evaluated. PCR-based strategies will work best for the most conserved genes, but not as well for evolutionarily divergent genes, or for genes specific to conifers. Efforts are now being devoted primarily to genes of practical interest, such as those involved in plant defense and wood-related properties.

Mapping cDNA's using RFLP's does permit an approximation of the chromosomal location of transcribed sequences which we hope to later refine as we elucidate basic features of conifer genome structure and evolution. In conifers, our use of probes that represent expressed genes (cDNA's) implies the construction of a transcriptional map; however, the unusually large and complex

nature of conifer genomes perhaps makes the more general term "gene map" a more accurate descriptor of the results our mapping efforts.

A loblolly pine gene map will be a valuable tool to forest tree geneticists. Access to the gene map will be made easy through the Dendrome database (Sherman and Neale 1993). Dendrome will integrate a continually expanding body of molecular and genetic information in an electronic format. The map will provide information about genome structure and organization. Genes identified and mapped could become valuable diagnostic tools for monitoring forest health in natural populations. For example, probes for genes that regulate drought tolerance or determine resistance to pathogens or insects could be used. In addition, gene probes will be useful for studying adaptive genetic variation in tree populations. Identifying and sequencing loblolly pine genes using heterologous probes will also provide insight towards sequence conservation between angio- and gymnosperms. Finally, the gene map will help provide the molecular foundation necessary to genetically manipulate forest trees using biotechnological approaches.

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SIMULATION STUDY OF LINKAGE MAP CONSTRUCTION WITH MISSING AND MIS-SCORED RAPD DATA

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Abstract. --Due to the recent interest in the random amplified polymorphic DNA (RAPD) technique for use in genetic linkage mapping, a series of computer simulations was conducted to investigate the effects of missing and mis-scored data on recombination estimates and linkage group construction. Ten maps (100 randomly distributed markers scored on 80 individuals) constructed with the software GREGOR, were modified to produce varying levels of missing (5%, 10%, 15%, and 20%) and mis-scored (1%, 2%, 4%, and 8%) entries. The distribution and levels of missing data were modeled after that found in actual RAPD data sets and mis-scored data was targeted to occur within specific markers. The resulting data sets were entered into MAPMAKER II and two-point recombination estimates and linkage group information were obtained. Analysis of variance was used to determine if there were significant differences among treatment means for the standard deviation of two point recombination estimates, number of framework markers, number of linkage groups, number of markers per linkage group, and number of marker order changes. Given no mis-scored data, significant differences among treatment means were not detected until a level of 20% missing data was reached. At this level, on average, 17.3% fewer markers could be placed into framework groupings. Given no missing data, significant differences among treatment means were not detected until a level of 4% mis-scoring was reached. At this level, on average, 28.6% fewer markers could be placed into framework groupings. The interaction between missing and mis-scored data was also investigated and not found to be significant. Based on these simulations, it is suggested that levels as high as 15% missing data and 2% mis-scored data can be tolerated during primary genetic map preparation.

Keywords: Genetic mapping, linkage, polymerase chain reaction, PCR, random amplified polymorphic DNA, RAPD

INTRODUCTION

The relative ease and speed with which large numbers of RAPD markers can be generated makes them extremely appealing for use in constructing primary genetic linkage maps. RAPD markers are generated by the use of single, randomly sequenced oligonucleotide primers and the polymerase chain reaction (Williams et al., 1990). A segment of DNA is amplified whenever two nucleotide sequences with high degrees of similarity to that of the primer occur

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within 2-3 Kb of one another on opposite strands of the template DNA. Repeated cycles of denaturation and extension result in the exponential amplification of the segment. Despite its conceptual simplicity the kinetics of the RAPD reaction are quite complex. Annealing temperature, degree of sequence similarity at priming sites, and primer competition all can affect the amplification of RAPD markers. In addition, when large numbers of RAPD reactions are being run on a daily basis, a small percentage of reactions fail. As a result, amplification inconsistencies could produce spurious data in the form of mis-scored individuals, and unless re-amplified, failed reactions would have to be recorded as missing data. The goal of this research was to investigate what effects various levels of missing and mis-scored RAPD data have on recombination estimates and linkage group construction.

MATERIALS AND METHODS

Ten known marker maps and corresponding data sets were constructed using the software GREGOR version 1.3 (Nick Tinker, McGill University). A configuration to model 10 pairs of chromosomes, 160 possible loci per chromosome and 1% recombination between adjacent loci, was chosen. A marker list consisting of 100 randomly distributed loci was defined for each map. The parents used for generating the mapping population were defined as follows: parent 1 was heterozygous (complete coupling) for all 100 marker loci, parent 2 was defined as being homozygous recessive for all 100 marker loci (tester). This coding arrangement would be similar to that used when constructing maps from haploid megagametophyte data. The mapping population consisted of 80 individuals.

In order to investigate the effects of missing data, five MAPMAKER II-compatible data sets were produced from each GREGOR data set. One represented the true data set and four represented various levels of missing data (5%, 10%, 15%, and 20%), for a total of 50 data sets. In order to determine how missing data should be targeted, we looked at the distribution of missing entries in actual RAPD data sets. Based on data generated for 2 different slash pines (Nelson et al., 1992; vanBuijtenen et al., 1992) and a longleaf pine (Kubisiak et al., 1992), missing data appear to be exponentially distributed. Most markers have no, or a few missing entries, with considerably fewer markers being found as levels of missing data increase. By randomly sampling from the function describing this distribution, the study targeted each marker to receive a specified number of missing entries, so that when averaged over all markers, the data set-wide levels were equal to 5%, 10%, 15%, or 20% missing.

Five MAPMAKER II-compatible data sets containing various levels of mis-scoring (0%, 1%, 2%, 4% and 8%) were also produced from each GREGOR data set, for a total of 50 data sets. When RAPD loci were scored, markers were categorized based on a confidence score (Kubisiak, et al., 1992). A putative polymorphic locus was given a lower confidence rating if the locus of interest was only faintly amplified or bands of similar molecular weight as the locus of interest were present. If mis-scoring were to result from one of these two sources, most errors should be occurring within specific loci, versus being random over the entire data set. Therefore, in order to produce the overall levels of 1%, 2%, 4%, and 8% mis-scoring, 20% of the markers were randomly chosen to receive 5%, 10%, 20%, or 40% mis-scoring.

The data sets were entered into the computer package MAPMAKER II (version 1.9), and recombination estimates and linkage group information were obtained. The mapping strategy was similar to that suggested by Lander et al. (1987). To determine all two-point groupings,

a log of the odds ratio (LOD) of 5.0 and a recombination frequency of 0.25 were chosen. To determine marker order within a particular linkage group, a LOD score of 3.0 was chosen. These markers, and their respective orders, were designated as framework groupings.

In order to evaluate the effects of missing or mis-scored data, an analysis of variance was used to determine if there were significant differences among treatment means for various descriptive measures. These included the standard deviation of the departure of two-point recombination estimates from their "true" or known values [$\text{std}(/r-\theta/)$], number of framework markers mapped, number of linkage groups obtained, number of markers per linkage group, and number of marker order changes. The $\text{std}(/r-\theta/)$ had the following form:

$$\sqrt{\frac{\sum_i^p \left[(r_i - \theta_i)^2 - \frac{\sum_i^p (r_i - \theta_i)^2}{p} \right]}{p}}$$

r = pairwise recombination estimate
 θ = "true" or known pairwise distance
 p = number of pairwise comparisons
 (For 100 loci $p = 4950$)

RESULTS

Given no mis-scored data, $\text{std}(/r-\theta/)$ was found to increase with the level of missing data (Table 1). A significant difference among treatment means occurred at 15%. With no missing data, $\text{std}(/r-\theta/)$ did not appear to increase until a level of 4% mis-scoring was attained (Table 1). However, it was not until 8% that a significant difference among

Table 1. Effect of missing and mis-scored data on the standard deviation of two-point recombination estimates [$\text{std}(/r-\theta/)$].

Missing Data		Mis-scored Data	
Treatment	Mean	Treatment	Mean
(% missing)	$\text{std}(/r-\theta/)$	(% mis-scored)	$\text{std}(/r-\theta/)$
0	0.056045 A*	2	0.055173 A
5	0.056627 A	1	0.055358 A
10	0.057566 A	0	0.056045 A
15	0.059567 B	4	0.056638 A
20	0.061033 B	8	0.060773 B

*Those means with the same letter are not significantly different at $\alpha=0.05$ using Tukey's Studentized Range Test. Means based on 10 replicate data sets.

treatment means was detected. The number of markers placed into framework groupings was found to decrease as the level of missing or mis-scored data increased (Table 2). A significant difference among treatment means was not detected until a level of 20% missing

data was attained. For the mis-scored data sets, a significant difference among treatment means was not detected until a level of 4% was attained (Table 2). Missing data did not appear to affect the number of linkage groups obtained in any sort of a

Table 2. Effect of missing and mis-scored data on the number of framework markers mapped (F.M.).

Missing Data		Mis-scored Data	
Treatment (% missing)	Mean # F.M.	Treatment % mis-scored)	Mean # F.M.
0	69.8 A*	0	69.8 A
5	68.6 A	1	66.5 A,B
10	65.0 A	2	60.7 B
15	64.6 A	4	49.8 C
20	57.7 B	8	48.3 C

*Those means with the same letter are not significantly different at $\alpha=0.05$ using Tukey's Studentized Range Test. Means based on 10 replicate data sets.

predictable manner (Table 3). No significant differences among treatment means was detected. However, as the levels of mis-scoring increased the number of linkage groups obtained was found to decrease (Table 3). A significant difference among treatment means occurred at 4%. The average number of markers per linkage group was, generally,

Table 3. Effect of missing and mis-scored data on the number of linkage groups (L.G.).

Missing Data		Mis-scored Data	
Treatment (% missing)	Mean # L.G.	Treatment (% mis-scored)	Mean # L.G.
15	14.8 A*	0	14.6 A
0	14.6 A	1	14.6 A
10	14.3 A	2	13.9 A
5	14.0 A	4	11.9 B
20	13.8 A	8	11.5 B

*Those means with the same letter are not significantly different at $\alpha=0.05$ using Tukey's Studentized Range Test. Means based on 10 replicate data sets.

found to decrease as the levels of missing or mis-scored data increased (Table 4). However, the differences among treatment means was more problematic as groupings overlapped. Finally, the number of marker order changes was not found to be affected by missing data in any sort of a predictable manner, and no significant differences among treatment means was detected (Table 5). The number of marker order changes was found to increase up to the level of 2% mis-scoring, after which numbers decreased again (Table 5). Treatment means were not determined to be statistically different.

Table 4. Effect of missing and mis-scored data on the average number of framework markers per linkage group (M/L.G.).

Missing Data		Mis-scored Data	
Treatment (% missing)	Mean # M/L.G.	Treatment (% mis-scored)	Mean # M/L.G.
5	4.948 A*	0	4.811 A
0	4.811 A,B	1	4.568 A,B
10	4.591 A,B	2	4.388 A,B
15	4.410 A,B	8	4.216 B
20	4.216 B	4	4.194 B

*Those means with the same letter are not significantly different at $\alpha=0.05$ using Tukey's Studentized Range Test. Means based on 10 replicate data sets.

The interaction between missing and mis-scored data was also investigated and not found to be statistically significant for any of the variables investigated (Data not shown).

Table 5. Effect of missing and mis-scored data on the number of marker order changes (O.C.).

Missing Data		Mis-scored Data	
Treatment (% missing)	Mean # O.C.	Treatment (% mis-scored)	Mean # O.C.
20	0.50 A*	2	1.20 A
10	0.30 A	4	0.80 A,B
0	0.20 A	1	0.50 A,B
15	0.10 A	0	0.20 A,B
5	0.00 A	8	0.10 B

*Those means with the same letter are not significantly different at $\alpha=0.05$ using Tukey's Studentized Range Test. Means based on 10 replicate data sets.

DISCUSSION

Prior to the analysis, we hypothesized that as levels of missing or mis-scored data increased within a data set the standard deviation of the departure of pairwise recombination estimates from their "true" or known values, $\text{std}(\hat{r}-\theta)$ would increase; likewise, the accuracy with which the genetic distance between two markers can be estimated decreases. The results seem to support our hypothesis. The $\text{std}(\hat{r}-\theta)$ was found to increase as the levels of missing or mis-scored data increased (significance at 15% and 8%, respectively).

We hypothesized that levels of mis-scored data would have a more pronounced effect on $\text{std}(\hat{r}-\theta)$ than would comparable levels of missing data. Missing data only indirectly affects linkage estimation by reducing the effective mapping population within particular markers.

However, mis-scored data would tend to confound the linkage relationship between markers and hence directly effect recombination estimates. Interestingly, these simulations indicate that, in terms of $\text{std}(r-\theta)$, the overall effects of a 5% level of missing data are comparable to a 4% level of mis-scoring.

The differences among mean values for the various levels of missing data and mis-scored data appear to be quite small in terms of genetic distance, however, this is due to the fact that a majority of the markers which are unlinked are still estimated to be unlinked even when harboring missing or mis-scored data. Therefore, a large number of the comparisons are not contributing to the sum of squared deviations in the calculation. The levels at which significant differences among means were detected does appear to be indicative of a problem threshold. In other words, the levels of missing or mis-scored data at which significant differences among treatment means were detected for $\text{std}(r-\theta)$ are similar to the levels found to cause significant group discrimination in other measures such as the number of framework markers, number of linkage groups, and number of markers per linkage group.

It makes sense that the number of markers placed into framework groupings would decrease as levels of missing and mis-scored data increase. These results indicate just such an inverse relationship (Table 2). Given no mis-scored data, at levels of 20% missing data, 17.3% fewer markers could be placed into framework groupings. Given no missing data, at levels of 4% mis-scoring, 28.6% fewer marker could be placed into framework groupings. In terms of the number of framework markers placed, lower levels of mis-scoring appear to have a more profound effect than do comparable levels of missing data.

Developing *a priori* hypotheses regarding how missing and mis-scored data might affect the number of linkage groups and average number of markers per linkage group was a more problematic situation. It could be hypothesized that missing or mis-scored data might cause whole linkage groups to fall apart, resulting in fewer mapped groups. Alternatively, mis-scored or missing data might cause larger groups to be broken into two or more smaller groups, resulting in a larger number of mapped groups, each having fewer markers. In terms of missing data, no apparent trends were found in the number of linkage groups obtained. However, as levels of mis-scoring increased, the number of linkage groups obtained decreased. This would seem to indicate that mis-scoring is primarily causing entire linkage groups to be lost. Although the number of framework markers per linkage group appears to decrease with increased missing or mis-scored data, no significant difference among treatment mean groupings were found.

Prior to the analysis, we hypothesized that marker ordering would be adversely influenced by increased levels of missing and mis-scored data. We also felt that, at comparable levels, mis-scored data would have more of an influence on marker ordering than would missing data. Over all simulated data sets, surprisingly few marker order changes occurred (38 or 0.38 per data set). Consistent with our *a priori* expectations, 27 (71%) of the changes were found to occur in mis-scored data sets. There does not appear to be any apparent trend in the number of marker order changes for the various levels of missing data. However, for the mis-scored data, the number of marker order changes increased up to the 2% level, beyond which they decreased. Low levels of mis-scoring tend to cause marker order changes, whereas higher levels confound linkages, causing markers to be dropped from the map.

Interestingly, there does not appear to be an additive effect when both missing and mis-scored data are included in the same data set. For example, with 10% missing data, the mean number of markers placed into framework groupings was 65, that is 4.8 fewer than with no missing, and with 2% mis-scored, the mean number placed was 60.7, 9.1 fewer than with no mis-scored. When both were included, the number of markers placed was 64.2. We would have expected this mean to be somewhat lower if the effect of both missing and mis-scored data was additive.

In addition to gaining a better understanding of how missing and mis-scored data effect primary map construction, we were also interested in how trends in the simulated data sets compared with those seen in actual RAPD data sets. Due to the fact that we do not know the true distances between markers or the level and distribution of mis-scoring in actual RAPD data sets, some of the measures investigated in this paper are not directly comparable. However, we do know the level and distribution of missing values. In a RAPD data set generated for longleaf pine, levels of missing data were found to approach 5%. When compared with simulated data sets with 5% missing data, some general trends appear. For the simulated data sets, on average, 68.6% of the markers could be placed into framework groupings. For longleaf pine, 64.3% of the markers (121 out of 188) were placed into framework groupings. In the simulated data sets, the majority of the markers that are lost as a result of 5% missing data are those harboring the highest levels of missing entries. Only 52.6% of the markers with greater than 15% missing entries mapped, whereas 71.4% of those markers with less than 15% missing entries mapped. For the longleaf data set, only 41.2% of the markers with greater than 15% missing entries mapped, whereas 66.1% of the markers with less than 15% missing entries mapped.

Although the amount of mis-scoring that occurs within an actual RAPD data set is not known, we felt that if mis-scoring were occurring it would be primarily concentrated in the markers with lower confidence scores. For the longleaf pine data set, 70.6% of the markers classified as good were mapped, whereas only 52.2% of the markers classified as fair were mapped. Mis-scoring might possibly be responsible for the difference between these percentages.

CONCLUSION

Based on the variables analyzed in this simulation study, it appears as if levels of missing and mis-scored data as high as 15% and 2%, respectively, can be tolerated during primary genetic map preparation, since they do not significantly effect recombination estimates or linkage group construction. The genetic system simulated in this analysis, 100 markers distributed randomly over a 1600 cM genome, is fairly representative of the situation encountered during the early phases of linkage mapping. We caution, however, that the results from this study are only applicable to low density mapping situations (i.e. early map construction). When more saturated linkage conditions exist the effects of missing and mis-scored data will have more of an impact, particularly on marker ordering.

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DEVELOPING CODOMINANT PCR MARKERS IN PINES

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Abstract.--Genetic markers which are easy to use and reveal abundant allelic variability provide powerful tools for many forest genetics applications. Because of their simplicity, methods using the polymerase chain reaction (PCR) are being developed for many plants. One class of PCR-based marker takes advantage of random amplification of polymorphic DNA (RAPD), but RAPD markers suffer several disadvantages related to their dominance and to their lack of comparability across pedigrees and between laboratories. To circumvent such problems, we are developing codominant PCR-based markers for pines. Our initial efforts have focussed on a subset of the cDNA clones previously used for constructing genetic maps. Nucleotide sequences from cDNA clones are used to design PCR primers for amplifying targeted fragments of genomic DNA from loblolly pine (*Pinus taeda* L.). Our pilot study demonstrates the feasibility of generating PCR markers in this way, but this method is complicated by the abundance of gene families in pine genomes.

Keywords: *Pinus taeda*, polymerase chain reaction, genetic map, genetic marker, small subunit

INTRODUCTION

Genetic mapping has been widely used in basic and applied studies of plants and animals. Among their many applications, genetic maps provide descriptive information on genome organization and they can assist in isolating and cloning genes of interest (Neale and Williams 1991). Genetic maps can also help to interpret data collected in population and evolutionary studies. Moreover, by tracking specific chromosomal segments from generation to generation, genetic maps and their associated markers are becoming particularly important in plant and animal breeding (Tanksley et al. 1989; Neale and Williams 1991). We are engaged in a long-term project to develop genetic maps and associated map-based technologies for pines (Devey et al. 1991; Neale et al. 1992). We have focused on loblolly pine because it is the mostly widely planted commercially important forest species in the United States. However, we are keenly aware of the demand for map-based applications in other pines and conifers. To-date, we have largely worked to develop genetic markers based on restriction fragment length polymorphisms (RFLPs) because they are reliable and informative, and because the information they provide is comparable across pedigrees and species.

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Many factors can influence selecting genetic markers for a given purpose. Isozyme markers cannot be surpassed for their cost, simplicity and ease of use, but they are limited to relatively small numbers of informative (i.e. polymorphic) loci. RFLPs are essentially unlimited in number, but intricacies associated with their use limits them to technically sophisticated laboratories. Recently, PCR based markers such as RAPDs (Williams et al. 1990, Welsh and McClelland 1990) have received much attention. Unfortunately, RAPD markers tend to be difficult to reproduce between laboratories and between pedigrees. Ignoring problems of reproducibility, RAPDs are typically dominant, so much of the high levels of heterozygosity found in forest tree species (e.g. Hamrick et al. 1981) cannot be detected unless haploid tissues (in conifers) are sampled or other special circumstances apply (e.g. Grattapaglia et al. 1992). Despite their obvious utility, RAPDs have limited applicability for forest trees.

Forest trees are different from nearly all other crop plants in that even the most commercially important species are relatively undomesticated. As was pointed out some time ago (Libby et al. 1969) this feature brings both opportunities and responsibilities. While we are very much interested in breeding applications stemming from our mapping efforts (e.g. Williams and Neale 1992), there is a need for technology to support conservation and stewardship activities on both public and private forests (e.g. Millar et al. 1990 and associated papers). DNA markers clearly have a role to play in this arena (Schaal et al. 1991, Wagner 1992). Ideally, such markers would be codominant, multiallelic, simple to use and assay, reproducible, reliable, and to the extent possible, useful in related species. Hence, given the breadth of the questions to address, and yet recognizing limited resources within the forestry community, we are striving to develop marker technologies that are simple to use and yet as broadly applicable as possible.

Most of our mapping efforts to-date have used RFLPs detected using anonymous cDNA clones. On average, 10-20% of these cDNAs about reveal mappable polymorphisms. A distinct advantage of using cDNA clones is their utility for identifying RFLPs in related conifer species (Neale and Williams 1991, Colby et al. 1993, and Ahuja et al., in press). This suggests that PCR primers designed on the basis of a cDNA from loblolly pine may also be able to amplify genomic DNA from related pines and perhaps other conifers.

An unusual feature of pine genomes, at least as compared to other higher plants, is the frequency and extent of multigene families (Gerttula and Kinlaw, 1993, and Kinlaw, unpublished). We postulated that such apparent complexity might be caused by genes with large or many introns (Kinlaw et al. 1990), but since a few conifer genes have now been characterized (Hutchison et al. 1990, Kojima et al. 1992, Spano et al. 1992, and Harry et al. in preparation) this now appears unlikely. Such features of conifer genomes are important to consider because the ability to amplify genomic DNA and to detect polymorphisms depends on the size and nature of the amplified PCR fragments.

We are now studying the feasibility of using PCR primers designed from cDNA clones to detect allelic variability in loblolly pine. Here we report the identification of a PCR-based polymorphism that maps to the same location as a RFLP marker detected the using same cDNA probe from which the PCR primers were designed.

METHODS

We selected a subset of 27 cDNA clones that had previously been used as probes to generate either of two genetic maps using RFLPs (Devey et al. 1991, Groover et al. 1993). Our primary selection criterion was that the clones revealed well-resolved RFLP banding patterns on Southern blots of loblolly pine genomic DNA. Typically, we selected clones

revealing few RFLP bands, but we also selected a few clones with more complex banding patterns (e.g. Gerttula and Kinlaw, 1993).

After selecting the clones, we determined the nucleotide sequence for 150-250 bases into each end of the cloned inserts (i.e. one sequencing reaction and gel, using standard dideoxy sequencing methods). Nucleotide sequences were then compared against published sequences contained in the GenBank electronic database to determine whether they appeared similar to any previously characterized sequences. If a tentative identification was made, then additional information was sought concerning the nature of the identified sequence and any corresponding genes that had been characterized. If the latter search found any plant genes with known gene structures (e.g. location and size of exons and introns), then this information was used to help determine suitable locations for PCR primers. Finally, we used the computer program PRIMER (version 0.5, M. Daly, S. Lincoln, and E. Lander, Whitehead Institute, unpublished) to assist in designing PCR primers. Generally, we choose primers to be 18-21 nt long, with a T_m near 60° C, and GC content of 40-60%.

Reaction conditions for the PCR amplifications were standardized after testing a range of concentrations for such components as template DNA and Mg^{++} (Perkin-Elmer protocols). Genomic DNA samples were prepared for the PCR using standard CTAB extraction procedures (Devey et al. 1991), supplemented by an additional organic extraction and ethanol precipitation. Final DNA concentrations were estimated with a fluorometer (Hoefer) using calf thymus DNA as a standard. The following conditions are described for 50 μ l reactions, but 25-100 μ l reactions were used with equal success after adjusting all components proportionately. Reactions were set up containing 30 ng of genomic DNA and 1.5 mM Mg^{++} in 40 μ l volumes (other components followed standard Perkin-Elmer recommendations) without *Taq* polymerase, overlain with mineral oil, and subjected to an initial denaturation step of 100°C for 5 min, then cooled to 85°C. At this point, 1.25 U *Taq* polymerase was added in 10 μ l of 1x PCR buffer supplemented with gelatin and DMSO for a 1% (v/v) final concentration. Immediately afterwards, temperature cycling began using the following conditions: 4 cycles of 1 min, 96° denaturation; 1 min, 55° annealing; 2 min 72° extension; followed by 30 cycles in which the denaturation step was lowered to 94°. A 5 min extension at 72° was added as the final step, and then the samples were cooled to 4°. PCR fragments were subjected to agarose gel electrophoresis in 1-2% agarose (BRL) and visualized by staining with ethidium bromide.

RESULTS AND DISCUSSION

Similarity Searches

Based on our sample of 27 cDNA clones, and including preliminary results from a larger sample (Colby et al. 1993, and Kinlaw, unpublished) we find that about 25% of the cloned cDNAs contain sequences that are similar (60% similarity or better) to previously characterized sequences. We also confirmed that two pairs of clones that were presumed to be similar to one another based on similar RFLP patterns on Southern blots indeed are similar at the level of nucleotide sequence.

Among the identified sequences we found two photosynthetic genes--those encoding the chlorophyll a/b binding proteins and others encoding the small subunit of ribulose biphosphate carboxylase (rubisco). The latter are represented by the cDNA clones pPt1IFG2025 and pPt1IFG1635 (henceforth abbreviated as 2025 and 1635). We now focus our attention on PCR products derived using primers derived from these clones.

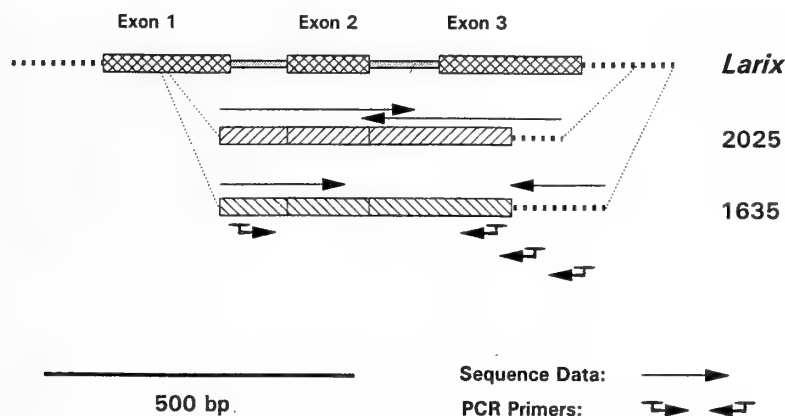
Small Subunit Gene Family

Nucleotide sequences for the small subunit of rubisco (rbcS) have been characterized from a variety of plants. RbcS genes typically belong to a small gene family (summarized by Dean et al. 1989) showing a conserved gene structure. Individual genes contain a sequence encoding a transit peptide, and most angiosperm genes contain two introns with identical locations (Dean et al. 1989). Small subunit clones have also been isolated from conifers, including a cDNA from *Pinus thunbergiana* (Yamamoto et al. 1988) and a genomic sequence from *Larix laricina* (Hutchison et al. 1990). The structure of the larch gene appears similar to angiosperm genes in that it encompasses a sequence for a transit peptide and also has two introns (Figure 1).

The nucleotide sequences from 2025 and 1635 were easily aligned with those from larch and with each other (Figure 1). The 5' ends of both cDNAs begin near the middle of the first exon in larch. The nucleotide sequences from the two sequencing reactions done for 2025 overlapped and so could be joined, but those for 1635 could not because it contains a longer insert. In fact, no 3' coding sequence was detected in 1635 using only the one sequencing reaction.

Four PCR primers were designed for the rbcS cDNAs using the following rationale. A single "forward" primer was chosen near the 5' end of the cDNAs in a region conserved in 1635, 2025, and also in larch (Figure 1). This is, therefore, a "generic" rbcS forward primer that may prove useful for amplifying DNA from a number of conifer species. In a similar vein, a reverse primer was selected near the 3' end of the coding sequence in 2025, also in a region conserved in pines and larch. In addition, two reverse primers were designed to correspond to non-coding regions unique to either 1635 or 2025. We reasoned that such 3' specific primers might preferentially amplify a subset of the rbcS gene family members.

Figure 1. Alignment of loblolly pine cDNAs pPt1IFG2025 and pPt1IFG1635 with a gene from larch. Two PCR primers (indicated by forward and reverse arrows) were designed to align with coding sequences, and two additional primers were designed to be specific to the non-coding regions at the 3' ends of the cDNA clones. Exons (representing coding regions) are wide boxes with cross-hatches. Introns are indicated by narrow boxes with stipples. Exons and introns (from larch) are drawn to scale.



PCR Amplification using RbcS Primers

As expected, PCR amplifications done using the forward and reverse coding sequence primers amplified both 1635 and 2025 cDNAs, as well as genomic DNA from loblolly pine. Only one fragment was seen from the genomic sample. The size of the PCR fragment from the genomic sample was about 220 bp longer than from the cDNAs, suggesting the presence of one or more introns with a total length of 220 bp. This is consistent with the larch gene, which contains two introns with a combined length of 203 bp. Furthermore, different members of the *rbcS* family, if they are indeed represented in this pool of PCR fragments, have very similar gene structures.

Both 3' specific primers also amplified genomic DNA, and the size of single PCR fragment from these reactions was consistent with expectations predicted in Figure 1: the 2025-specific 3' primer produced a fragment of about 780 bp, whereas the 1635-specific 3' primer produced a larger fragment, about 870 bp. To determine whether the 3' specific primers might selectively amplify different *rbcS* genes, both were used for PCR reactions using both 2025 and 1635. The 2025-specific primer only produced a PCR fragment using 2025 template, and likewise for the 1635-specific primer. Even so, it appears that both 3' specific primers amplify more than one gene since restriction digests of the amplified products reveal DNA fragments whose combined size is larger than the undigested products (problems associated with incomplete digests can be excluded).

Allelic Segregation

To identify allelic polymorphisms, PCR fragments were generated using all three pairs of *rbcS* primers to amplify genomic templates from individuals in a 3-generation pedigree (Devey et al. 1991). No length polymorphisms were observed among samples of undigested DNA. To reveal variation in nucleotide sequence, the PCR fragments were subjected to digestion using an array of restriction enzymes with 4-base recognition sequences. To-date we have found one polymorphism that segregates in a Mendelian fashion.

PCR fragments generated using the 3' primer specific to 2025 were digested with *Bst*NI. Most of the PCR fragments produced in this way lacked a *Bst*NI site, but for one of the four grandparents of the pedigree, this digestion produced two novel fragments, of about 360 and 420 bp (i.e. summing to the 780 bp of the undigested product). The F_1 parent produced by this grandparent also contained this fragment, as did 7 of the 21 tested F_2 progeny. In other words, variation for the presence of a *Bst*NI site appears to behave as a Mendelian marker.

Because of the allelic configuration in this cross, we have not yet observed an individual that is homozygous for the allele carrying the *Bst*NI site. However, because of the nature of the *rbcS* gene family, we believe that such an individual would have a phenotype identical to a heterozygote. That is, the 780 bp fragment would still be present because it also represents PCR fragments that were generated from other genes, and these products also lack the restriction site. We plan to test this prediction by observing the PCR phenotypes of haploid gametophytes from seeds of the heterozygous parent.

Co-segregation with a 2025 RFLP Marker

To determine whether this PCR-based marker appears linked to the mapped 2025 RFLP marker, we cross-classified individuals whose phenotypes had been scored for both. For the RFLP marker, the parents had been classified as A1A2 x A1A3, and we observed

the four genotypic classes segregating approximately 1:1:1:1 (Figure 2). Only two PCR phenotypes could be distinguished, but the "b" allele (carrying the *Bst*NI site) was completely coincident with the allele A3 (Figure 2). These data suggest that the RFLP allele A3 is linked with the PCR-marker, and indeed probably represents a DNA fragment with an internal *Bst*NI site that the A1 and A2 RFLP alleles lack. To our knowledge, this is the first example whereby a mapped RFLP polymorphism has been converted to a mapped PCR-based polymorphism in conifers.

Figure 2. Cross-classification of RFLP and PCR genotypes among individuals segregating for an *rbcS* polymorphism. RFLP genotypes were scored from Southern blots probed using the loblolly pine cDNA clone pPt1IFG2025. PCR genotypes were scored using PCR primers designed from the same cDNA clone.

PCR Genotypes		RFLP Genotypes					
			A1 A1	A1 A2	A1 A3	A2 A3	Total
		aa	10	4	0	0	14
		ab	0	0	3	4	7
		Total	10	4	3	4	21

Parental Genotypes:

RFLP: A1 A2 x A1 A3

PCR: aa x ab

We are encouraged by these results in many respects. We have learned a great deal about the nature of the *rbcS* gene family in pine with a few relatively simple experiments. For example, the size of the fragments produced by digesting the *Bst*NI suggests that the polymorphic site is contained in the second intron. Base substitutions are expected to be more frequent in introns because of the constraints imposed by natural selection in coding sequences. Moreover, by selecting PCR primers in non-coding regions flanking expressed genes we can reduce the molecular complexity in the pool of amplified products, and thereby simplify genetic interpretations for any polymorphisms that are observed.

However, we have seen that it may be a difficult task to detect length polymorphisms among PCR fragments generated using primers from expressed genes, at least unless specific steps are taken to do so. Nevertheless, we are also encouraged by the results of Morgante and Olivieri (1993), who located a number of simple sequence repeats in the introns of plant genes. Allelic variants of simple sequence repeats contain different numbers of repeat units, and therefore represent length variants. Experimental strategies designed to identify such repetitive sequences in the introns of loblolly pine genes may prove to be productive. We are about to begin such a series of experiments.

CONCLUSIONS

This preliminary survey leads us to the following tentative conclusions:

1. PCR primers selected from sequencing mapped cDNAs can reveal useful and mappable genetic polymorphisms.
2. Polymorphisms that reveal themselves as length variants among PCR fragments are not common. Instead, other methods are needed to detect variation nucleotide sequence among the fragments. Such methods could be as simple as restriction digests, but more powerful techniques may also be required.
3. Gene families in pines will complicate gene interpretations if multiple members of the family are amplified simultaneously.

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Genetic Mapping of Quantitative Trait Loci Influencing Wood Specific Gravity in Loblolly Pine (*Pinus taeda*).

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Abstract.--We are attempting to map quantitative trait loci affecting wood specific gravity in a three-generation loblolly pine pedigree. Forty-eight progeny were measured for wood specific gravity and genotyped for 178 restriction fragment length polymorphism markers. Each marker was tested for linkage to variable genetic factors which influence wood specific gravity by comparison of progeny marker genotype class means for wood specific gravity. Fourteen markers showed differences in genotypic class means at the 0.01 level. An additional 127 progeny were genotyped and analyzed for one of the 14 significant markers. The marker, Pt1IFG149a, accounted for 5% of the total phenotypic variation in the progeny for wood specific gravity.

Introduction

Phenotypic variation of quantitative traits such as height, insect and disease resistance, diameter, and wood specific gravity can be partitioned into additive and non-additive genetic and environmental components. Such partitioning describes the collective effects of all variable genes which influence the phenotype. The opportunity exists, using molecular markers and genetic maps, to identify and characterize individual genetic factors which influence quantitative traits, hereafter referred to as quantitative trait loci (QTL).

Quantitative trait loci have been identified for numerous traits in several crop species. Edwards *et al.* (1987) identified QTL for each of 82 traits evaluated in two F₂ populations of maize. Individual QTL accounted for between 0.3% and 16% of the total phenotypic variation for a given trait, and the cumulative effects of all QTL explained between 8% and 40% of the total phenotypic variation per trait. Dominant and overdominant gene action at individual QTL was prevalent, especially for yield-related traits. QTL were also identified which influenced trait stability. Stuber *et al.* (1992) showed that F₂ maize plants which were heterozygous for QTL alleles influencing grain yield had higher yields than individuals homozygous at the same locus. The authors hypothesized

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overdominant gene action at these QTL and concluded that the QTL play a role in explaining heterosis. There was little evidence for interactions of QTL with environments in this study, despite replication across diverse environments.

QTL which influenced fruit size, soluble solids concentration, and pH were identified in tomato for progeny of a cross between *Lycopersicon esculentum* (cultivated tomato) and *L. cheesmanii* (a related wild species) (Paterson *et al.* 1991). The trial, conducted in three environments, detected numerous QTL X environment interactions, as only four of the 29 QTL detected had a significant effect in all three environments. There was evidence for QTL with pleiotropic effects. As with maize (Edwards *et al.* 1987), digenic epistasis was not common between QTL.

Wood specific gravity is an important determinant of wood quality and influences lumber quality and pulp yield. Heritability estimates for wood specific gravity are generally high and there is abundant additive genetic variation segregating in advanced generation pedigrees of several conifers. These factors make wood specific gravity a good trait for QTL mapping (Williams and Neale, 1992).

A linkage map has been constructed for loblolly pine (*Pinus taeda* L.) based on RFLP and isozyme markers using a single, three-generation pedigree (Devey *et al.*, in preparation). We report here the use of similar marker technology to locate and characterize QTL which influence wood specific gravity in loblolly pine.

Methods

Pedigree selection

A three-generation, full-sib loblolly pine pedigree with extreme wood specific gravity (WSG) values within grandparental pairs and a high variation for WSG in the F₂ progeny was available from the Weyerhaeuser Company (Figure 1) (Williams and Neale, 1992). A total of 175 progeny trees on six sites, four in North Carolina and two in Oklahoma were available for analysis.

WSG measurement

Radial cores were taken for each progeny at the approximate center of the internode below breast height. Each core was cropped at the pith and at the outer edge of the ring boundary corresponding to age eight. Wood specific gravity was determined on an oven-dry weight, green volume basis.

RFLP and Isozyme mapping

Forty-eight progeny with extreme WSG values (24 lowest and 24 highest) were selected for genotyping with RFLP and isozyme markers. Selecting individuals with extreme quantitative trait value minimizes the number of individuals that must be genotyped for a given power of detection of QTL (Lander

and Botstein, 1989). Loblolly pine complementary DNAs (cDNA) were the main source of probes for producing RFLPs (using methods described by Devey *et al.* (1991)). In addition, several cloned genes from loblolly and Scots pine, random loblolly pine genomic clones, and random radiata pine genomic clones were used as probes. Four isozyme loci (Gdh, Got2, Mdh3, and Sk) were scored. Linkage analysis was performed using GMendel 2.0 (Liu and Knapp, 1990).

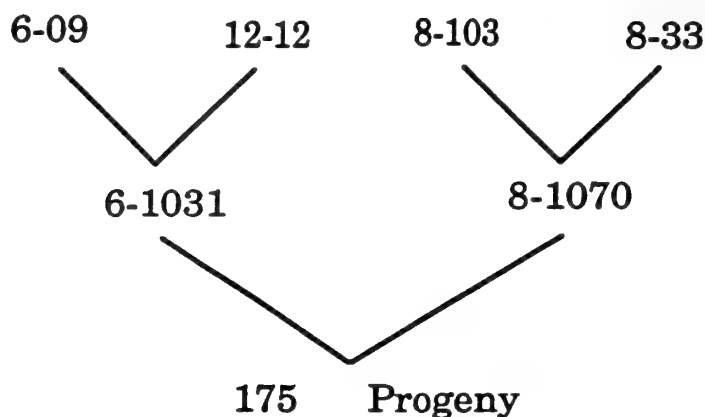


Figure 1. Three-generation loblolly pine pedigree for mapping wood specific gravity QTL.

Marker/QTL association

For a given RFLP marker, progeny were grouped based on their RFLP genotype. Individuals within a group had in common the region of parental DNA associated with the marker alleles, while regions of DNA unlinked to the marker varied at random among them. The effect of unlinked regions of DNA thus averages to zero within each group, and comparison of mean WSG among the groups tests for the effect of the region of DNA associated with the marker on WSG. Significant differences in mean WSG among the groups is evidence for the presence of a QTL residing in close proximity to the marker. A prerequisite for detecting a WSG QTL in this experiment is for one or both parents to contain alternative alleles for the QTL which differ in their effect on WSG. Incomplete linkage results in the misclassification of individuals with respect to QTL genotype whenever crossing over between the marker and QTL occurs. Consequently, the ability to detect marker/QTL cosegregation decreases with increasing distance between the marker and QTL. Markers with three or four alleles and for which parental trees are both heterozygous are the most informative. Such markers allow the estimation of the influence of both male and female alleles at that locus on WSG.

We are using two approaches to detect linkage between markers and QTL. The first is an analysis of variance approach which, for each marker, compares the mean WSG among progeny RFLP genotype groups. Significant differences in mean WSG among groups indicates linkage between the marker and a QTL. The

data for each marker was analyzed using SAS Proc GLM using the model shown in Table 1. Marker genotype was considered a fixed effect, while site and the site X marker genotype interaction were considered random effects.

Table 1. Effects in ANOVA model for testing differences in marker genotypic classes means.

Source	df	Expected Mean Squares
Site	s-1	$s^2_e + tms^2_s$
Marker	m-1	$s^2_e + ts^2_{s*m} + Q(m)$
Site*Marker	(s-1)(m-1)	$s^2_e + ts^2_{s*m}$
error	n-s-m-1	s^2_e

The second approach under development uses interval mapping as described by Lander and Botstein (1989). This approach uses the information from flanking markers and thus uses more of the information contained in the marker data. The interval mapping software is currently being tested and no results from this method will be presented in this paper. The analysis of variance and interval mapping approaches will eventually be compared for ability to detect QTL.

Results

WSG measurements

Mean WSG for all progeny was 0.3868 (standard deviation=0.0181). The distribution was approximately normal ($W=0.97$).

Genetic Map

To date, 178 RFLP loci have been scored and mapped for the subsample of 48 progeny. Most of the RFLPs were produced using loblolly pine cDNAs. Scots pine Lhc and Sod clones, and loblolly and radiata pine random genomic clones all produced mappable RFLPs. The map, as constructed by GMendel 2.0, is composed of 14 linkage groups of three or more markers and nine linkage groups of two markers; sixteen markers are presently unlinked.

QTL/Marker associations

The effect of genotype was significant ($p<0.01$) for 14 of the 178 RFLP markers tested using the analysis of variance approach. For these 14 markers, the remaining 126 progeny are currently being genotyped. We report below the results from a single marker (marker Pt1IFG149a, revealed by a loblolly pine cDNA) for which genotyping of all progeny has been completed and for which a basic analysis has been performed.

Marker 149a

Marker 149a is fully informative in the genetic sense as both parents are heterozygous for marker alleles and it is possible to discern the parental contribution to the marker genotype of all progeny. The genotype of the parent trees and the resulting four genotypic classes in the progeny for marker 149a are shown in Figure 2.

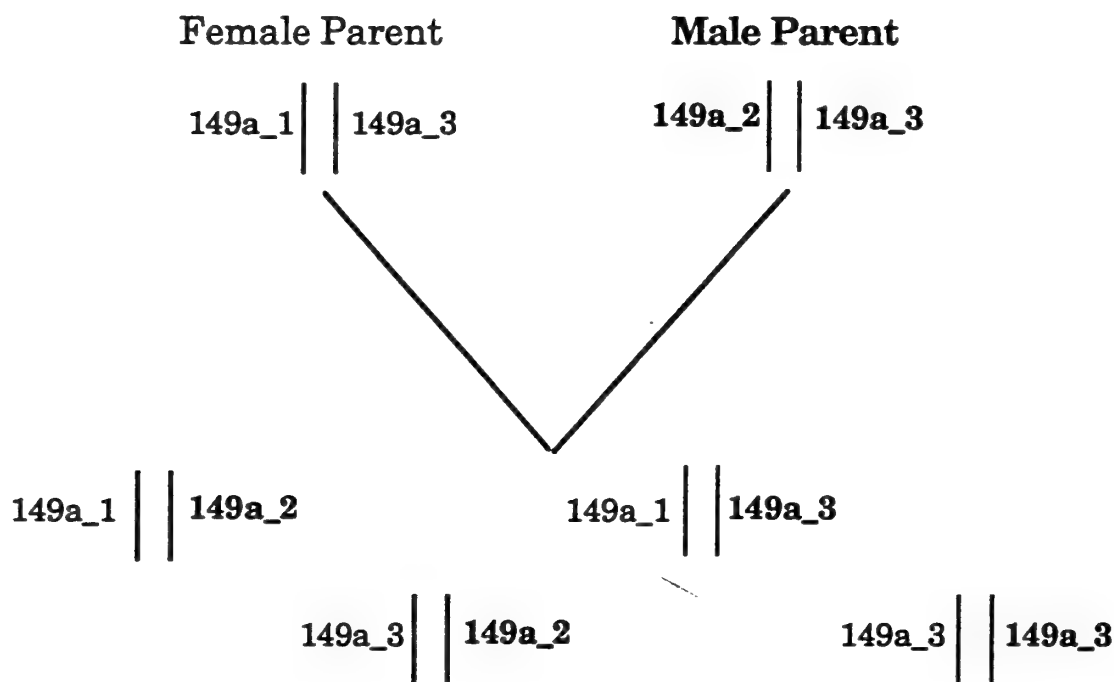


Figure 2. Parental genotypes for marker 149a and resulting four genotypic classes in the F2 progeny. Male marker alleles are indicated in **bold**.

Modeling linkage phase of marker and QTL alleles

The first step of the analysis is to determine whether one or both parents are segregating at the quantitative trait locus. The mean WSG of progeny receiving the 1 marker allele from the female parent (mean WSG=0.3844, SE=0.0023) is lower than the mean WSG of the progeny receiving the 3 marker allele from the female parent (mean WSG=0.3892, SE=0.0022). The resulting hypothesis is the female parent is heterozygous for QTL alleles of alternative effect, and that the 1 female marker allele is in coupling phase with a QTL allele which decreases WSG, while the 3 female marker allele is in coupling with a QTL allele which increases WSG. Similarly, on the male side of the cross, the mean WSG of the progeny receiving the 3 marker allele from the male parent (mean

WSG=0.3841, SE=0.0016) is lower than the mean WSG of the progeny receiving the 2 marker allele from the male parent (mean WSG=0.3898, SE=0.0015). The resulting hypothesis is the male parent is also heterozygous for QTL alleles of alternative effect, and that the 2 male marker allele is in coupling with a QTL allele which increases WSG and the 3 male marker allele is in coupling with a QTL allele which decreases WSG. Note that the 3 marker allele is apparently associated with QTL alleles of opposite effect in the two parental trees. This is not a surprising result considering the high degree of linkage equilibrium present in coniferous tree populations, and illustrates one of the interesting aspects of QTL mapping in conifers.

Testing the proposed model

Based on the apparent effect of parental QTL alleles, progeny were allocated to three groups (Figure 3). The first group contained those trees receiving an RFLP allele coupled to a QTL allele which increases WSG from both parents, and were thus homozygous for QTL alleles increasing WSG (assuming no crossing over between the marker and QTL). A second class was formed by those progeny receiving an RFLP allele from each parent coupled to a QTL allele which decreases WSG, and were thus homozygous for QTL alleles which decrease WSG. The remaining progeny received a QTL allele from one parent which increases WSG and a QTL allele from the other parent which decreases WSG, and thus formed a class heterozygous for QTL alleles of opposite effect.

The means of the three groups were tested for significant differences by ANOVA (Table 2). The effect of genotype was highly significant; the F value is probably of less certainty than the $Pr > F$ of 0.002 given by Proc GLM in SAS because of heterogeneity of variances among the classes of genotypes within environments. Closer examination of the data reveals that the trees receiving the 1 female RFLP allele and the 2 male RFLP allele have a distinctly higher variance at four of the six sites when compared to the other 3 RFLP progeny classes. Although this complicates interpreting the results of the ANOVA, it is suggestive of a QTL influencing trait variability, as described by Edwards et al. (1987). We found no evidence for interaction of QTL alleles with environments.

Variance component estimates for the effects in the model are shown in Table 3. The percent of total phenotypic variation explained by the marker genotype was 5%, as estimated by dividing the variance attributed to genotype by the sum of the variance components for genotype, site*genotype, and error. The negative value associated with the site*genotype variance was replaced by zero in this calculation.

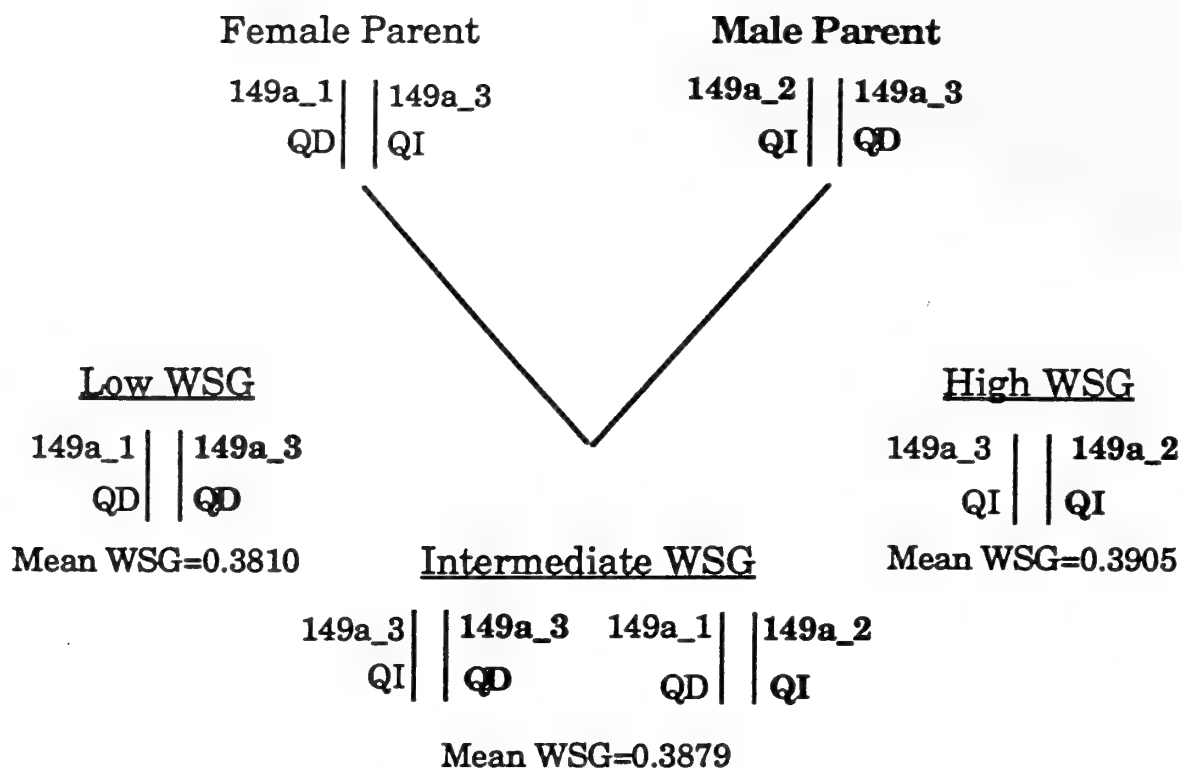


Figure 3. Segregation of marker 149a and linked QTL, assuming complete linkage. QTL alleles are described by QI (increasing WSG) or QD (decreasing WSG).

Table 3. ANOVA for marker 149a. The correct test of the effect of genotype in this mixed model (site random, genotype fixed) is MS Genotype divided by MS Site*Genotype.

<u>Source</u>	<u>df</u>	<u>Mean Square</u>	<u>Pr>F</u>
Site	5	0.00072340	0.0389
Genotype	2	0.00101597	0.0019
Site*Genotype	10	0.00008091	0.9867

Table 3. Variance component estimates for factors in ANOVA model, as produced by Proc Vcomp (SAS). All effects were considered random for this analysis.

<u>Variance component</u>	<u>Estimate</u>
(Var)Genotype	0.00001499
(Var)Site	0.00002733
(Var)Site*Genotype	-0
(Var)Error	0.00029969

Discussion

We have identified at least one QTL influencing wood specific gravity in a three-generation loblolly pine pedigree, demonstrating that it is possible to identify QTL for a coniferous tree species. Given the pedigree structure and modest sample size of this experiment, the fact that QTL were detected indicates that there exist variable genes with major effect on wood specific gravity. This finding parallels the results of QTL experiments in other plant species, where QTL with differing magnitude of effect have been detected.

Conifer populations are characterized by a high degree of genetic diversity. This is evident at the DNA level from the high frequency of restriction fragment length polymorphism revealed by loblolly pine DNA probes. Often, probes reveal multiple mappable RFLP loci and up to four alleles may be segregating at individual loci in the progeny generation. This differs from backcross or F2 populations where only two alleles can be segregating at any given locus. To simplify the analysis, we presented a reduced genetic model in which QTL alleles were classified as increasing or decreasing WSG. While the simplified model is adequate for detecting the presence of a QTL, it does not describe the interaction among QTL alleles at a locus. A given combination of QTL alleles could interact in an additive, dominant, or overdominant fashion. We plan to expand the analysis to examine the interaction between QTL alleles at a given locus, as well as possible digenic epistatic effects between pairs of QTL.

The full progeny set has been genotyped and analyzed for only one of the 14 markers which gave significant results. However, the results from that analysis illustrates some interesting points. The high degree of linkage equilibrium in coniferous populations was illustrated by marker allele 3 being linked to QTL alleles of different effect in the two parental trees. Also, the data from this marker suggest that QTL which influence trait variability may exist for WSG. This is not a surprising result given similar findings for various traits in maize (Edwards *et al.* 1987).

As we identify additional QTL influencing WSG, we hope to further characterize them with respect to gene action and digenic epistasis. Also, we will

be testing other loblolly pine pedigrees for variability in the same QTL detected in this experiment. Because of the high degree of genetic diversity present in conifers, only some of the QTL detected in this experiment would be detectable in a different cross involving unrelated trees. Likely, additional QTL will be detected in these experiments. Eventually, we will also test QTL identified for ontogenetic effects.

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HIGH DIVERSITY BUT LITTLE POPULATION SUBDIVISION OF A LODGEPOLE PINE CHLOROPLAST DNA POLYMORPHISM

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Abstract.--We have surveyed a chloroplast DNA restriction fragment length polymorphism in a total of 365 individuals, distributed rangewide in eight natural populations of lodgepole pine (*Pinus contorta* Dougl.). Six variants were found in the survey, and diversity was high ($H_{es} = 0.44$). Population subdivision was weak within and among subspecies (e.g., $F_{st} = 0.04$ among subspecies), which is consistent with theoretical predictions for paternally-inherited markers in wind-pollinated outcrossers. Comparison of the chloroplast data with mitochondrial frequency data from the same populations provides evidence that gene flow is differential through seeds and pollen.

Keywords: *Pinus contorta* Dougl., mitochondria, RFLP, paternal inheritance, differentiation, genetic variation.

INTRODUCTION

An understanding of the amounts and patterns of genetic variation is fundamental not only for tests of evolutionary hypotheses, but also for effective genetic improvement and germplasm conservation strategies. In plants, this requires consideration of variation in three genomes: chloroplast, mitochondrial, and nuclear.

Theoretical analyses have anticipated that genetic subdivision among populations is sensitive to mode of inheritance. For example, maternally inherited cytoplasmic variation in plants is expected to exhibit greater differentiation at equilibrium than nuclear genes, as a consequence of (i) organellar migration through seeds but not pollen, and (ii) the diploid nuclear but haploid organellar composition of seeds (Birky 1988; Petit 1992). In fact, chloroplast polymorphisms in *Quercus* L. (Whittemore and Schaal 1991; Petit et al. 1993) and *Datisca* L. (Liston et al. 1992), as well as mitochondrial polymorphisms in pines (*Pinus* L.) (Dong and Wagner 1993; Strauss et al. 1993), do indeed display substantial population differentiation (with F_{st} values as high as 0.895).

In contrast, population subdivision of paternally inherited loci, because of their migration through both seeds and pollen, is expected to be much weaker than that of maternally inherited factors in outcrossing plants (Petit 1992). Examples of paternally inherited chloroplast DNA (cpDNA) polymorphisms are known in several plant taxa including conifers (Schumann and Hancock 1989; Boblenz et al. 1990; Wagner 1992), but empirical estimates of population differentiation for these types of polymorphisms are not available in the primary literature. Here

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we report the estimation of population subdivision parameters of a paternally inherited cpDNA polymorphism in lodgepole pine (*Pinus contorta* Dougl.).

METHODS

Plant Materials

The germplasm collections have been described in detail elsewhere (Dong and Wagner 1993). Briefly, a total of 365 individuals were sampled from eight natural populations of lodgepole pine (Table 1). Sampled populations were distributed rangewide and included three of the four subspecies. The available samples provide approximately 95% power to detect variants that occur (i) with frequency ≥ 0.07 within populations, and (ii) with frequency ≥ 0.008 in the total survey.

Table 1. Sample site locations (abbreviations in parentheses), taxonomic classification, and sample sizes^a.

Location	Taxonomic Classification	Sample Size
Mackenzie, British Columbia (BC-N)	<i>P.c.l.</i>	44
Prince George, British Columbia (BC-C)	<i>P.c.l.</i>	43
Lumby, British Columbia (BC-S)	<i>P.c.l.</i>	43
Ward, Colorado (CO)	<i>P.c.l.</i>	49
Prince Rupert, British Columbia (BC-W)	<i>P.c.c.</i>	43
Waconda Beach, Oregon (OR-W)	<i>P.c.c.</i>	46
Santiam Pass, Oregon (OR-C)	<i>P.c.m.</i>	47
Wrights Lake, California (CA)	<i>P.c.m.</i>	50

^a Map locations are shown in Dong and Wagner (1993). Key to taxonomic classification: *P.c.l.* = *P. contorta* var. *latifolia*; *P.c.c.* = *P. contorta* var. *contorta*; *P.c.m.* = *P. contorta* var. *murrayana*. For discussion of taxonomic classification of OR-C population, see Wheeler and Guries (1982).

Laboratory Methods

In general, chloroplast sequences evolve slowly (Clegg et al. 1991), and interparental chloroplast recombination is very rare or nonexistent (Chiu and Sears 1985). Thus, many chloroplast haplotypes (plastomes) should be identifiable by the genotypes of any sufficiently variable cpDNA polymorphism. With this in mind, we elected to examine a single highly variable polymorphism, rather than several markers of lower variability. This choice maximized the numbers of individuals and populations that could be surveyed, given the available resources. The particular polymorphism chosen for this study was selected because (i) it was the only cpDNA polymorphism known to vary within lodgepole pine, and (ii) its paternal inheritance has been demonstrated (Dong et al. 1992).

Each sampled individual was classified by genotype (variant) of an *Sst*I restriction fragment length polymorphism (RFLP), as described previously (Wagner et al. 1987; Dong et al. 1992). However, in the present study a 7.4-kilobase-pair (kbp) *Hind*III fragment and a 700-base-pair *Bam*HI-*Sma*I fragment from the lodgepole pine chloroplast genome (Lidholm and Gustafsson 1991) were used interchangeably as probes in molecular hybridizations. The *psbA* gene is duplicated in lodgepole pine (Lidholm et al. 1991), and either of these probes reveal insertion/deletion polymorphism associated with the *psbAI* - *psbAII* genomic region. This polymorphism may be due to copy-number variation of short tandem repeats (Lidholm and Gustafsson 1991) and is a "hot spot" of chloroplast variation in lodgepole pine (Wagner et al. 1987; Govindaraju et al. 1989).

Data analysis

Frequency data from the eight populations (Table 2) were used to estimate: numbers of variants in species (A_s) and populations (A_p), unbiased variant diversities in species (H_{es}) and populations (H_{ep}), and population differentiation (F_{st}) (Wright 1951; Nei 1978; Hamrick and Godt 1990; Weir 1990, p. 150). The statistical significance of differentiation was evaluated by chi-square (Weir 1990, p. 137).

RESULTS AND DISCUSSION

Diversity

Six variants were found in the total survey. The number of variants per population ranged from two to five with an average of four. Diversities within populations ranged from 0.26 to 0.66. Notably, no population was fixed for a single cpDNA variant (Tables 2, 3).

Chloroplast DNA variability in lodgepole pine, whether measured in terms of variant number or diversity (Table 3), is greater than that of typical isoenzyme polymorphisms in this and other plants (Wheeler and Guries 1982; Hamrick and Godt 1990). This may appear remarkable but does not contradict the generally slow rate of chloroplast sequence evolution (Clegg et al. 1991), for at least two reasons.

Table 2. Chloroplast genotypic frequencies in 8 populations of *P. contorta*^a.

Variants ^b	Populations							
	var. <i>latifolia</i>				var. <i>contorta</i>		var. <i>murrayana</i>	
	BC-N	BC-C	BC-S	CO	BC-W	OR-W	OR-C ^c	CA
4.3/5.0	0.07	0.12	--	0.04	0.19	0.11	0.02	0.02
4.4/5.0	0.09	0.14	0.16	0.08	0.09	0.13	0.09	0.04
4.5/5.0	0.73	0.65	0.84	0.86	0.53	0.61	0.79	0.86
4.7/5.0	0.11	0.09	--	--	0.14	0.13	0.11	0.08
4.3/4.5/5.0	--	--	--	--	0.05	0.02	--	--
4.3/4.7/5.0	--	--	--	0.02	--	--	--	--

^a Location abbreviations are defined in Table 1 (see also Dong and Wagner 1993).

^b Variants are denoted by restriction fragment sizes (in kilobase pairs); only the variable fragments are listed, separated by slashes within each variant.

^c See Wheeler and Guries (1982) for discussion of subspecies taxonomic classification in this geographic region.

First, the *psbA*-associated polymorphism results from insertions/deletions, possibly due to the presence of short tandem repeats (Govindaraju et al. 1989; Ali et al. 1991; Lidholm and Gustafsson 1991). Such repeats have been implicated in generating high levels of cpDNA variation in other plants (Palmer et al. 1987; Aldrich et al. 1988; Blasko et al. 1988; Ogihara et al. 1988). Clearly, data from polymorphisms that arise through length mutation are unrelated to conclusions about chloroplast base-pair substitution rates.

Second, recall that we chose to study a single hot spot of cpDNA polymorphism, precisely because of *a priori* knowledge of its intraspecific variability (Wagner et al. 1987). Thus, this polymorphism hardly portrays the situation for "typical" chloroplast base pairs. Several other population surveys of cpDNA diversity, including those that have examined point mutations, have also focused on polymorphic hot spots (e.g., Whittemore and Schaal 1991; Petit et al. 1993). Consequently, much of the accumulating cpDNA population data is not representative of random base pairs.

Despite these caveats, intraspecific cpDNA hot spots carry useful information. When such markers occur, their high diversity permits efficient assay of chloroplast haplotypes. This diversity, combined with uniparental inheritance (either maternal or paternal), empowers new fields of inquiry, such as cytonuclear population genetics (Asmussen et al. 1987).

Table 3. Population genetic statistics in *P. contorta*^a.

Statistics ^b	Populations							
	var. <i>latifolia</i>				var. <i>contorta</i>		var. <i>murrayana</i>	
	BC-N	BC-C	BC-S	CO	BC-W	OR-W	OR-C ^c	CA
A_p	4	4	2	4	5	5	4	4
H_{ep}	0.46	0.55	0.28	0.26	0.66	0.60	0.37	0.26
Mean A_p	4							
Mean H_{ep}	0.43							
A_s	6							
H_{es}	0.44							
F_{st}	0.04 (among subspecies); $p < 0.001^d$							
	0.02 (within var. <i>latifolia</i>); $p < 0.05^d$							
	-0.01 (within var. <i>contorta</i>); N.S. ^d							
	-0.01 (within var. <i>murrayana</i>); N.S. ^d							

^a As in Table 2.^b Abbreviations for population genetic statistics are defined in the text.^c As in Table 2.^d Chi-square probabilities.

Differentiation

Two of the chi-square tests indicate statistically significant ($p < 0.05$) frequency differences among sampled populations and subspecies (Table 3). Nonetheless, the plastome differentiation among populations and subspecies is weak: all F_{st} values are 0.04 or less, and pairwise genetic identities (Nei 1978) range from 0.94 to 1.00 (the genetic identity matrix is available upon request from the authors). Weak differentiation is consistent with theoretical expectations for paternally inherited polymorphisms in outcrossers (Petit 1992).

In contrast, population subdivision of maternally inherited mitochondrial polymorphism, *estimated from the same DNA samples that we used for the chloroplast analysis*, is much higher (e.g., $F_{st} = 0.31$ among subspecies, and F_{st} is as high as 0.82 among populations within subspecies, Dong and Wagner 1993). We note that, given sufficient intraspecific variability, the maternally inherited cpDNA polymorphisms typical of other plants also generally exhibit considerable subdivision among populations (reviewed by Soltis et al. 1992).

Interestingly, the CO population was fixed for a "private" (Slatkin 1985) mitochondrial variant (Dong and Wagner 1993), yet all but one individual in this population had cpDNA variants typical of other lodgepole pine populations (Table 2). A Colorado population also differed from more central populations in an isoenzyme study (Wheeler and Guries 1982). Taken together, this information is consistent with a dearth of seed migration but occasional pollen migration involving Colorado populations, which are located at the periphery of lodgepole pine's current distributional range. Similarly, the high frequency of two private mitochondrial variants in the OR-W population (Dong and Wagner 1993) contrasts with the cpDNA data (Table 2), again compatible with differential gene flow through pollen and seeds.

CONCLUSIONS

Pines clearly represent an unusual model system for population and evolutionary genetic investigations, because of their opposite chloroplast and mitochondrial inheritances (Neale and Sederoff 1989). In lodgepole pine, population subdivision of the three major eukaryotic genomes conforms with theoretical predictions for outcrossers. Specifically, Mendelian allozymes and paternally inherited plastomes display little differentiation among populations (Wheeler and Guries 1982; this study, Table 3), while maternally inherited mitochondrial polymorphisms feature abundant population subdivision (Dong and Wagner 1993). Knowledge of the population genetic architectures of differentially-inherited plant genomes may be useful when biologists choose genetic markers for specific purposes.

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COMPLEX PINE GENE FAMILIES:

C. S. Kinlaw ^{1/} and S. M. Gerttula ^{2/}

Abstract. We have initiated a long term study to determine the molecular mechanisms which have driven pine genome evolution. Pine genomes are large and contain many examples of repeated sequences suggesting that pine genome evolution has included repeated duplication or amplification events. Our initial studies reported here characterize the structure of two classes of repeated sequences. One class revealed by copy DNA probes encodes structural proteins and the second class revealed by a genomic DNA clone is a retrotransposable element. By comparing the structure of complex gene families within the genomes of two distantly related pines, loblolly pine and western white pine, we can determine evolutionary pathways for specific gene families and begin to infer molecular mechanisms which have operated upon pine genomes over geological time.

Keywords: *Pinus taeda*, *Pinus monticola*, loblolly pine, western white pine, complex gene families, molecular evolution, DNA, Southern hybridization

INTRODUCTION

The goals of forest tree molecular genetics research include obtaining an understanding of the organization of DNA sequences within the genomes of existing tree species as well as developing models of the molecular mechanisms by which forest tree genomes have evolved over geological time. We have chosen to focus our attention upon pines as they represent an economically and environmentally important genus containing a biologically and genetically diverse group of modern species. *Pinus* is an ancient genus with roots in the Mesozoic era more than 136 million years ago (see review Millar and Kinloch, 1989), and an important aspect of pine genome evolution appears to have been the repeated duplication or amplification of individual DNA sequences to form complex families. The existence of such complex families and the mechanisms by which they arise and are maintained within pine genomes may have important consequences for how individual pine genes function.

Pine genomes are large and contain high percentages of repeated sequences which encode no known structural proteins, RNAs or regulatory function. Published reports of genome sizes range from 22 pg/nucleus for Monterey pine to 49 pg/nucleus for maritime pine (Govindaraju and Cullis, 1991). Loblolly pine, a focus of our current studies contains 22 pg/nucleus (Dillon, 1987). Data for the size of the western white pine genome, also a focus of our current studies, is not currently available in the literature. Molecular comparisons to other pines (Ahuja *et al.*, submitted for publication, Kinlaw and Gerttula, unpublished) suggest that western white pine is larger than loblolly pine and similar in size to sugar pine (40 pg/nucleus).

For comparison to a variety of angiosperms, corn contains 10 pg/nucleus, rice contains 2 pg/nucleus, poplar 2 pg/nucleus, peach contains 1 pg/nucleus, *A. thaliana* 0.6 pg (Arumuganathan and Earle, 1991). Although some angiosperm genomes are also large, it is important to bear in

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mind that unlike angiosperms, pines show no evidence of genome duplications (polyploidy, Mirov, 1967). The chromosome number of all existing pine species is $2n = 24$.

Murray *et al.* (1981) developed a model for angiosperm genomes which may help explain the size of large pine genomes. According to this model, turnover rates determine genome size where turnover is defined as the sum of amplification and deletion. Low amplification rates or high deletion rates yield small genomes. Rapid amplification without rapid deletion yields large genomes containing small fractions that appear as "single copy" and large fractions of "fossil repeats" that have diverged in sequence following amplification. High amplification rates increase the probability that secondary amplification events (where adjacent elements are amplified together) will produce repeat heterogeneous families (Bendich and Anderson, 1977) composed of subfamilies which have been amplified at different times and which show different amounts of sequence divergence. Reassociation kinetics data (Kriebel, 1985) support the above model for pines. It appears that the pine genome is composed of repeated sequences present in a continuous range of frequencies with very few "low copy" sequences. Kriebel (1985) hypothesized that much of this low copy DNA may be the result of ancient transposable elements which diverged following amplification to such an extent that their DNA sequences no longer share significant similarity.

Even at the level of structural genes, the pine genome is complex. Random copy DNAs (cDNAs) from loblolly pine messenger RNA (mRNA) used as markers to develop restriction fragment length polymorphism (RFLP)-based genetic maps (Devey *et al.*, 1991) rarely reveal simple banding patterns indicative of single genes, but rather more often reveal a complex band pattern reflecting the presence of multiple copies of specific DNA sequences within pine genomes (Devey *et al.*, 1991). Angiosperms also contain complex gene families (plant genome I, November 1992) but not to the extent found in pines. In addition, a number of specific genes, for example alcohol dehydrogenase (Kinlaw *et al.*, 1990), lipid transfer proteins (Kinlaw and Gerttula, MS in preparation), and glutamine synthetase (Kinlaw and Gerttula, unpublished data) are present at several copies in crop species such as corn or rice and are present in many more copies in pines. Because the number of pine genes residing in complex gene families and the relative complexity of pine gene families represent an extreme in the variation observed for plant genomes, pines represent a unique opportunity to develop an understanding of the impact sequence amplification has had upon plant genome evolution as well as to develop an understanding of the impact complex gene families have upon the regulation of individual gene family members. We present here initial data which describes the structure and organization of two classes of repeated DNA sequences within pine genomes, one class consists of moderately repeated structural protein genes from the functional 10% of the pine genome and the other class consists of a highly repeated retrotransposon from the "nonfunctional" 90% of the pine genome.

METHODS

Genetic Materials

Loblolly pine needles were obtained from Westvaco (clone 3). Western white pine needles were obtained from Berkeley Botanical Garden (V10, row 34, line 26).

Probe Isolation

Complementary cDNA probes were used to identify RFLPs in structural protein gene families. The cDNA library was prepared from total RNA isolated from 12-day-old loblolly pine seedlings as reported by Devey *et al.* (1991). A genomic probe for the IFG element was isolated (Kossack, 1989) from a Monterey pine genomic library according to methods previously published (Harry *et al.*, 1989) and used to identify RFLPs in the IFG family.

DNA Procedures

Loblolly pine and western white pine genomic DNA was isolated from needle tissue by a modification of the method of Wagner *et al.* (1987) as described previously (Devey *et al.*, 1991) with the additional steps of subcellular fractionation to isolate nuclei. Following tissue grinding, filtering, and collection of a crude cellular pellet the samples were resuspended in extraction buffer containing 50 mM TrisHCl, pH 8.0; 5 mM MgCl₂, 350 mM sorbitol, 1% Triton X100; and 0.1% beta mercaptoethanol to lyse organelles. Nuclei were collected by brief centrifugation (2500g, 5 min.) and resuspended in buffer containing 50 mM TrisHCl, pH 8.0; 5 mM EDTA; 350 mM sorbitol, 0.1% beta mercaptoethanol. To lyse the nuclei, N-lauryl sarcosine was added to 1%, NaCl to 710 mM, and hexadecyltrimethylammonium bromide to 0.1%. Organic extraction with chloroform and DNA precipitation with ethanol followed the procedure outlined in Devey *et al.* (1991). Genomic DNAs were digested with two restriction enzymes (*Eco*RI and *Hind*III), subjected to electrophoresis, and blotted onto Zetaprobe (Biorad) membrane as described (Devey *et al.*, 1991). Hybridization was performed in roller bottles following the membrane manufacturers recommendations.

RNA Procedures

Poly A+ RNA from loblolly seedlings (Devey *et al.*, 1991) was hybridized to ³²P-labeled cDNA probes according to previously reported methods (Alosi *et al.*, 1990).

RESULTS AND DISCUSSION

Random cDNAs

Southern banding patterns of random clones show varying degrees of complexity (See figure 1). From a survey of 153 cDNA probes used for the RFLP mapping project (Devey *et al.*, 1991; Neale and co-workers), 29% hybridize to greater than 10 bands and are therefore considered to reflect complex gene families, while only 18% hybridize to a single band.

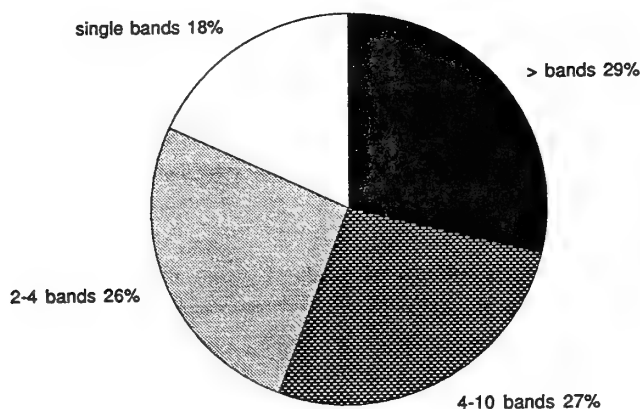


Figure 1. Frequency of cDNA classes
Southern blots from random cDNAs used for the RFLP mapping project (Devey *et al.*, 1991; Neale and co-workers) were surveyed and divided into four classes based upon the number of bands hybridizing to each probe.

We have chosen 10 structural protein gene families for further analysis. As shown in table one, we have determined the cDNA lengths and confirmed that each cDNA represents a functional gene by northern analysis. The DNA sequences of the chosen cDNAs have been determined and compared to databases. Only two share sufficient sequence identity to sequences in Genbank to allow for tentative gene identification. Clone 2027 appears to be derived from a lipid transfer

protein gene family, and 2022 appears to be derived from the glutamine synthetase gene family. Complex gene families pose significant challenges for mapping (Devey *et al.*, 1991), and the genome location for only a few of the members of complex gene families can be determined using RFLP methods. Of our chosen gene families, genomic locations for one or more loci have been determined for the 2068, 975, 2022, and 2027 gene families (Devey *et al.*, manuscript in preparation; Groover *et al.*, manuscript in preparation).

Table 1. cDNA Probes

cDNA clone	cDNA size bp	mRNA size Kb
pPt1IFG107	507	0.9
pPt1IFG846	562	1.8
pPt1IFG975	483	2.3
pPt1IFG1605	457	1.7
pPt1IFG1764	591	2.9
pPt1IFG1930	479	1.4
pPt1IFG1970	903	2.0
pPt1IFG2022	975	1.6
pPt1IFG2027	483	1.0
pPt1IFG2068	382	0.7

We present here (figure 2) the Southern banding patterns of 4 cDNA clones which span the range of complexity observed for loblolly cDNAs. Copy numbers within the loblolly genome can be estimated from copy number controls which we routinely include on our Southern blots. As can be seen from the autoradiograms (figure 2), band intensities vary. Some bands (e.g., lanes a and c, panel A, figure 2) are of the same or greater intensity as our 5-copy equivalent control and may represent tandemly repeated DNA sequences within the loblolly genome while other bands are of an intensity similar to the 1-copy equivalent control.

Most clones reveal a simpler pattern in western white pine than in loblolly pine with fewer and less intense bands (e.g., lanes b vs. d, panel A, figure 2). Southern hybridization depends upon DNA complementarity. Clones from loblolly are expected to differ in sequence from western white pine genes due to sequence divergence over time, and are therefore expected to hybridize less efficiently to western white pine genes than to loblolly genes. Clone 1930 is an extreme case where western white pine shows a much simpler banding pattern than does loblolly. (See panel B, figure 3.)

A notable exception to our general trend is 2022. Our loblolly probe reveals a more complicated and stronger signal in western white pine than in loblolly. (See panel C, figure 2.) This result strongly suggests that there has been more amplification of 2022-like sequences in western white pine than in loblolly. We conclude, therefore, that the mechanism for DNA amplification was still active in pines after the initial split of the pine genus into hard and soft pines. It is possible that this mechanism is still active in modern pine genomes, although we have no specific evidence of recent amplification events.

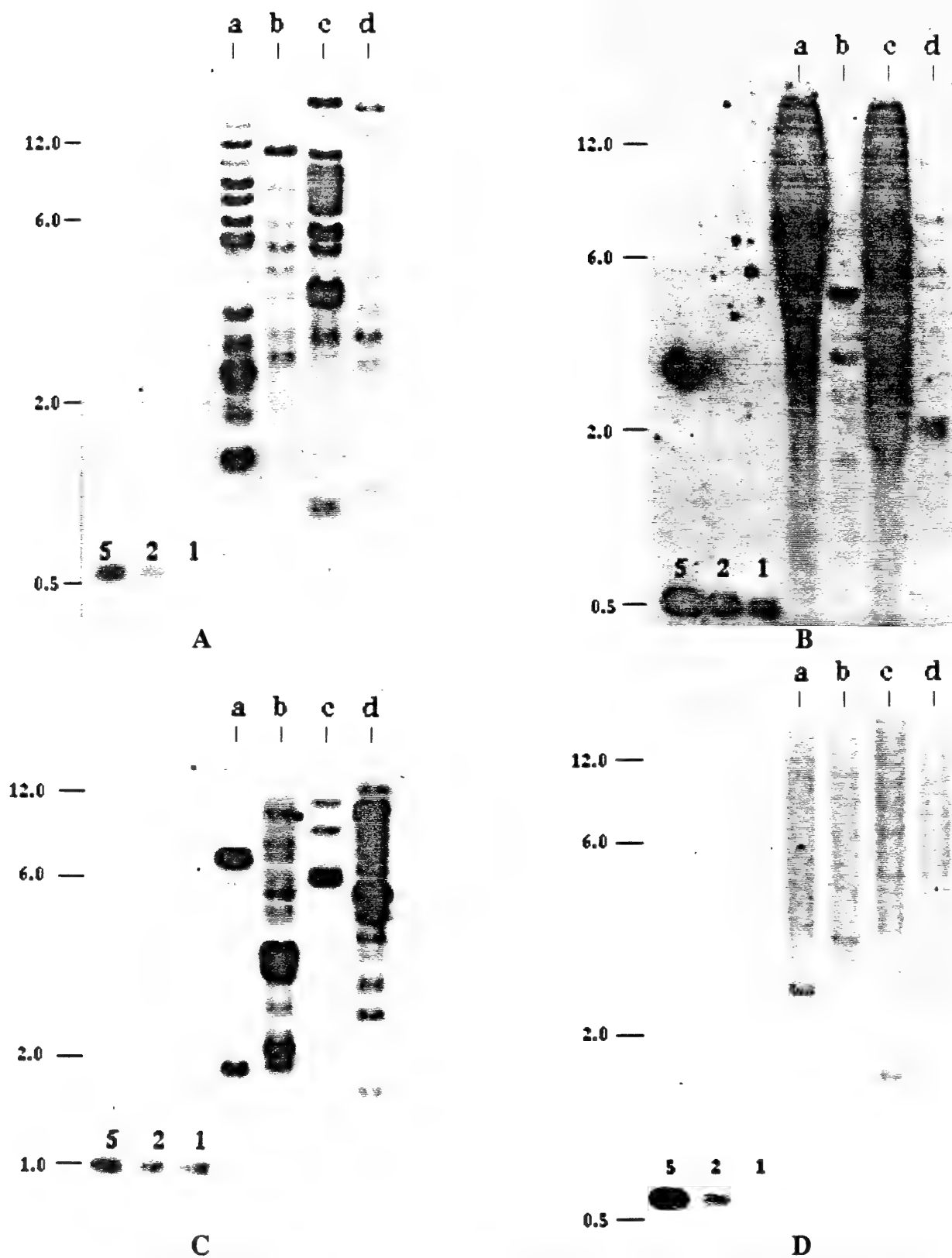


Figure 2. Autoradiograms of loblolly and western white pine DNAs hybridized with loblolly cDNA probes. Panel A probed with pT1IFG2027. Panel B probed with pT1IFG1930. Panel C probed with pT1IFG2022. Panel D probed with pT1IFG107. Lanes labeled 5, 2, and 1 in each panel are copy number equivalents per loblolly genome for each cDNA clone. Lanes a and c in each panel are loblolly DNA digested with *Hind*III and *Eco*RI respectively. Lanes b and d are western white pine DNA digested with *Hind*III and *Eco*RI respectively.

IFG element

In early experiments (Sederoff, unpublished data) to identify, isolate, and characterize highly repeated DNA sequences from pine genomes we stumbled upon the IFG element (Kossack, 1989), a DNA sequence which has the structure of a class of transposons called retrotransposons. Transposons encode the ability to amplify themselves and jump to new genome locations, hence the popular term "jumping genes". Transposons are termed "selfish" because they serve no known function for their host. Retrotransposons (see review Rubin, 1983) "jump" via an RNA intermediate and encode reverse transcriptase, the enzyme which synthesizes a DNA copy of the RNA intermediate thus allowing for integration of a new copy of the retrotransposon within its host genome. Retrotransposons are similar to and evolutionarily related to retroviruses but lack viral coat protein genes. Retrotransposons have been found in the genomes of animals, fungi, and plants; and there is some suggestion that they are capable of horizontal transmission between species even though they lack viral protein coat genes. The IFG element is to our knowledge the only known example of a conifer "jumping gene". Properties of the IFG element and other retrotransposons include:

1. Long terminal repeats (LTRs) of several hundred nucleotide base pairs at either end, each flanked by a pair of short inverted repeats. These long terminal repeats are the initiation sites for the synthesis of the retrotransposon RNA replication intermediate.
2. Coding regions for proteins which replicate and integrate new copies of the retrotransposon. These coding regions include the gag gene required for binding of the replication primer, reverse transcriptase which synthesizes a DNA copy of the RNA replication intermediate, and an integrase which cleaves the host DNA allowing for integration.
3. Random and dispersed integration sites throughout the host genomes and short duplications of the host target sequences.

The IFG element is approximately 6000 base pairs long and appears most similar to the *del* retrotransposon from lily (Smyth *et al.*, 1989). Based upon copy estimates from Southern hybridizations (data not shown) the IFG family is present in greater than 10,000 copies in all pines tested, including 38 species from the major subsections of the genus *Pinus*. Thus, the IFG element family represents approximately 0.5% of the entire pine genome. We have not detected an active IFG element within pines. To date, specific IFG elements sequenced contain numerous stop codons, and we have not detected IFG mRNA. Without isolating all 10,000 IFG copies from pines, we cannot rule out that one or more elements remain active with the ability to replicate new copies.

Figure 3 shows a Southern blot of loblolly pine and western white pine hybridized to an IFG sequence probe. As compared to the protein coding genes previously described, the IFG family members show more sequence conservation, e.g. a 1.0 Kb *Eco*RI band is present in both loblolly pine and western white pine IFG elements. (See figure 3 arrows.) However, the IFG element family does show considerable polymorphisms between the two pines, eg. a prominent *Eco*RI 1.5 Kb band is present in loblolly pine but not western white pine. (See figure 3 arrows.) The signal from loblolly pine is stronger than the signal from western white pine. This result is not surprising as the probe used for this Southern is an IFG element isolated from Monterey pine which is more closely related to loblolly pine than to western white pine.

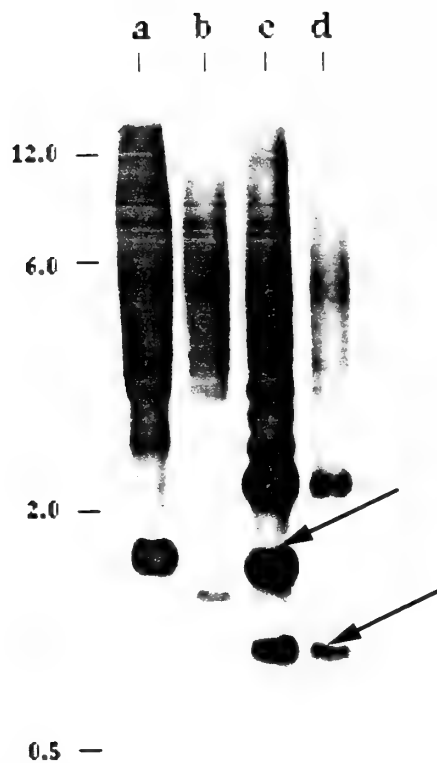


Figure 3. Autoradiogram of loblolly and western white pine genomic DNA hybridized to a Monterey pine genomic clone for IFG. Lanes a and c are loblolly DNA digested with *Hind*III and *Eco*RI, respectively. Lanes b and d are western white pine DNA digested with *Hind*III and *Eco*RI, respectively.

A simple model which might explain the high copy number and RFLP variation observed for the IFG family includes the following:

1. Horizontal transmission of an IFG progenitor into a pine ancestor.
2. Amplification of the original IFG progenitor element numerous times and dispersal to new genome sites.
3. Sequence divergence of individual IFG elements.
4. Amplification of modified elements (producing new RFLPs)
5. Continuation of 1-4 without significant deletion rates for amplified IFG elements.

Modern species whose ancestors diverged prior to the amplification of a particular IFG subfamily will have different IFG RFLPs, while RFLPs shared by a group of related pines reflect IFG elements whose sequences have not changed since the divergence of the species.

Transposition frequencies of retrotransposons are typically low but may increase in response to genome shocks such as cell culture or bridge-breakage-fusion cycles as seen in maize (Potter *et al.*, 1979). The genetic effects of retrotransposons as reviewed by Rubin (1983) are thought to have strong implications for the evolutionary processes of genomes and thus the divergence of species. Insertions can cause mutations of target genes by disrupting the integrity of transcription units or proteins. Mutations can be either quantitative or qualitative, and host suppressor genes can modify mutation phenotypes. Neighboring host genes can also be affected by retrotransposon insertions. Introducing an element carrying a strong promoter can alter local chromosome structure. Transcription which initiates within the right LTR of an element can continue into downstream host genes. Thus, levels and developmental profiles of host expression can be

altered. In addition to insertion events, recombination between LTRs can occur (Roeder and Fink, 1983). Recombination between LTRs within the same element results in the deletion of an element, and recombination between LTRs on different elements results in the deletion of intervening host DNA.

SUMMARY AND CONCLUSIONS

In summary, we conclude from our study of the IFG family and structural protein gene families that:

Pine genomes are complex containing amplified copies of different classes of DNA.

Retrotransposon families are more highly amplified than structural protein gene families.

Structural protein gene families have undergone significant sequence divergence, show varying degrees of amplification, and specific families can differ in complexity among pines.

This degree of fluidity at the DNA sequence level is in contrast to the high degree of conservation of chromosome number and size among pines.

Conifer chromosomes are known for their poor uptake of stains specific for condensed, repetitive DNA regions such as in C-banding (Borzan, 1988). In addition, ribosomal repeats show a more dispersed distribution within pine genomes than their angiosperm counterparts (Cullis *et al.*, 1988). Taken together with the high copy number and dispersed location of IFG retrotransposon elements, these results suggest that perhaps pines, in contrast to angiosperms, have more dispersed than tandemly arrayed repetitive DNA.

FUTURE DIRECTIONS

Many unanswered questions about the structure, function, and evolution of complex gene families remain. For example, what is the mechanism of amplification of pine sequences? The IFG retrotransposon most likely to have been amplified via an RNA intermediate, the mechanism termed retrotransposition. However, we do not yet know the mechanism of amplification of structural protein gene sequences. The method of amplification could well determine if the amplified copies are functional. To address this question, we plan to investigate the structure of genomic sequences for several clones and to determine the sequence of the 3' end of a number of cDNAs for a specific complex gene family. Because the 3' end of mRNAs are less conserved than the coding regions, we expect more sequence heterogeneity at the 3' end of mRNAs from a specific gene family if multiple family members are expressed.

Another important question to answer is whether only specific kinds of genes are amplified in pines or whether the amplification is random. We have initiated a project to sequence a number of cDNAs (Colby *et al.*, 1993). Once the identity of a number of complex gene families is accomplished from comparisons to database sequences, we may be able to discern patterns in what genes appear to be amplified. A related question is to what extent have genes been differentially amplified in different pine lineages. We have initiated a study to isolate western white probes to use in Southern hybridizations, and we plan to look for further examples like clone 2022 which show a different degree of amplification in western white pine and loblolly pine.

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SESSION 6

General Genetics

CONCEPTS RELATED TO INCREASED SUSCEPTIBILITY OF LOCAL SOURCES
OF LOBLOLLY PINE TO SOUTHERN PINE BEETLES

H.R. Powers,¹ R.P. Belanger,¹ W.D. Pepper,¹ and F.L. Hastings²

Abstract.--In a planting near Aiken, SC, loblolly pine saplings from an eastern seed source were significantly more susceptible to the southern pine beetle (SPB) than were loblolly saplings from western sources. In California, heavier infestations of the fir engraver beetle were reported on local than on distant sources of white fir. Such results conflict with the notion of native hosts and pests evolving together and developing mutual tolerance. The SPB case and the general concept deserve additional attention.

Keywords: Southern Pine Beetle, resistance, loblolly pine, fusiform rust

INTRODUCTION

The southern pine beetle (*Dendroctonus frontalis* Zimm.) (SPB) is one of the most devastating pests of southern pines. Silvicultural guidelines have been developed to recognize high-risk stands and to provide growing conditions that promote resistance to insect damage (Belanger 1980, Nebeker et al. 1985). Another approach, which has received relatively little attention, is to plant stock that has inherent resistance to SPB damage. It is well known that southern pine species differ in their susceptibility to SPB attack. Since there are differences in susceptibility among southern pine species, there also could be differences within a species.

Genetic selection in tree improvement programs has increased individual tree growth, stand yield, and product quality in pine plantations. In addition, highly fusiform rust resistant seedlings are now available that

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reduce losses to fusiform rust, caused by Cronartium quercuum (Berk.) Miyabe ex Shirai f. sp. fusiforme. While certain families of loblolly pine (Pinus taeda L.) have potential resistance to SPB attack (Nebeker et al. 1991), there are no SPB-resistant seedlings available for planting, nor have means been developed for large-scale testing for resistance to the SPB. There appear to be opportunities for making genetic gains through indirect selection, since oleoresin properties of individual trees vary within species as well as between species (Hodges et al. 1977, Nebeker et al. 1988, Nebeker et al. 1991). Proposals have also been made to study seasonal variations in resin flow from bark wounds as indicators of resistance to SPB (Lorio et al. 1990). In this paper, we report findings which indicate that the susceptibility of loblolly pines to SPB attack varies among seed sources and/or geographic provenances (Powers et al. 1992), and discuss their implications related to other insect problems.

MATERIALS AND METHODS

Design

The study area, a 7-acre tract in Aiken County, SC, was divided into 28 quarter-acre plots. Each plot was assigned one of seven geographic seed sources of pine. This area was originally a study designed to evaluate the resistance of various seed sources to fusiform rust. Five of the sources were loblolly pine and two were slash pine (P. elliotii Engelm. var. elliotii). Three of these seed sources from the western part of the loblolly range have been shown to be resistant to fusiform rust (Wells and Wakeley 1966): Clark County, AR; Angelina County, TX; and Livingston Parish, LA. The two eastern sources of loblolly pine were: (1) a mix of the most rust resistant families from the cooperative USDA Forest Service-Georgia Forestry Commission (USFS-GFC) rust resistant orchard in Milledgeville, GA, and (2) a control lot from a first-generation production orchard in central Georgia. Most of the parents providing seed for the latter two loblolly sources originated in Georgia and South Carolina. Two eastern sources of slash pine were also included in the study. They were a rust-resistant mix of seed from the USFS-GFC orchard, and a control lot from a first-generation production orchard in central Georgia.

The study design was a randomized complete block with four replications. Each replication contained 7 treatments (seed sources), each with 120 trees. Trees were planted in 1978 on an 8 x 10 ft spacing (544/ac). There were no border trees between the four blocks or the treatment plots. No silvicultural treatments, such as thinning or fertilization, had been carried out on these plots.

The relative rust resistance of these sources was reported 7 years after planting by Powers and Matthews (1987). When the trees were 11 years old, several SPB infestations were observed in the study area. Numbers of living and dead trees on each plot and the mortality caused by the SPB were recorded. No information was available on the origin or sequence of SPB attack, spot

proliferation, or spot spread. Detailed information was taken on the rust infections on a subsample of 30 trees within each quarter-acre plot to evaluate potential correlations between susceptibility to fusiform rust and SPB mortality.

SPB mortality per plot was calculated as the percentage of standing trees that had been killed by SPB. The dbh of all live trees was also measured. Rust incidence on the subsample of 30 trees in each plot was calculated as the percentage of all standing trees that had at least one stem gall. The average number of stem galls per infected tree and rust severity expressed as a percentage of the stem circumference encompassed by the most severe stem gall on the tree were also measured.

Statistical Analyses

Arcsine transformations of SPB mortality and rust incidence percentages were performed, and the six plot responses were used as dependent variables in a multivariate analysis of variance performed with SAS procedure GLM (SAS Institute Inc. 1987). This analysis simultaneously tested for the presence of a seed-source effect on all dependent variables and for the presence of partial correlations among SPB mortality and rust-related responses. Since it has not been previously demonstrated that there is a seed source (provenance) effect on resistance or susceptibility to a given SPB population, we elected to be very conservative in selecting a critical level of significance for F tests of contrasts. The comparison-wise error rate (CER) for judging F tests ¹/_c was calculated with Sidak's procedure (Games 1977): $CER = 1 - (1 - 0.05)^{1/c}$, where c = number of comparisons (five in this paper) and 0.05 is the maximum experiment-wise error rate. Our CER was 0.0102, rather than the traditional CER of 0.05.

RESULTS

A tree-by-tree survey for SPB mortality was made in all 28 plots of the study area during May 1990. The average proportion of trees killed by SPB varied from 5.3% for the Livingston Parish loblolly to 41.3% for the rust-susceptible check loblolly from Georgia and South Carolina (Table 1). The multivariate test of the simultaneous effects of seed source on arcsine of SPB mortality, arcsine of rust incidence, number of galls per tree, number of trees per acre, dbh of live trees, and percentage of stem girdled by most severe stem gall was significant. The approximate value of F (d.f. 36/47) was 3.84 (P = 0.0001). This statistic shows that the probability of the observed seed source effects occurring by chance is extremely small.

Table 1. SPB mortality, rust characteristics, and stand condition for seven loblolly and slash pine seed sources.

Seed source	SPB mortality	Fusiform rust ¹			Stand	
		Incidence	Galls	Severity ²	Trees	dbh
	(%)	(%)	No./ tree	(%)	No./ac	in.
Loblolly (western)						
Livingston Parish, LA	5.3	19.0	1.2	33	260	7.0
Clark County, AR	9.4	4.2	1.2	48	424	6.6
Angelina County, TX	6.5	1.5	1.0	35	416	6.9
Loblolly (eastern)						
Rust-resistant (USFS-GFC)	22.5	20.8	1.7	39	436	6.6
Commercial orchard (control)	41.3	34.2	1.5	36	432	6.8
Slash (eastern)						
Rust-resistant (USFS-GFC)	6.4	21.0	2.0	55	376	6.7
Commercial orchard (control)	6.6	51.0	2.1	50	368	6.6

¹Value based on stem infections only.

²Severity based on percentage of stem circumference girdled by most severe rust infection.

Multivariate analysis of variance including all sources failed to show any significant partial correlations ($P > 0.2$) between transformed SPB mortality and any of the rust- or stand-related responses. We concluded, therefore, that differences in rust susceptibility were not responsible for the observed variations in SPB mortality among seed sources. This result permitted us to proceed with univariate analyses for the five planned comparisons among seed sources.

Mortality was less than 10% for each of the three western sources of loblolly pine (Table 1), and differences among these three were not significant. As expected, SPB mortality was also low for the two eastern sources of slash pine. In comparing each of the eastern sources individually with the average of the western sources, the eastern loblolly commercial control sustained significantly more SPB mortality than the collective western sources ($P = 0.0004$) (Table 2). There was no statistically significant difference between the eastern loblolly rust-resistant source and the western

sources of loblolly pine in SPB mortality. The difference in SPB mortality between slash pine and the loblolly pine control was significant ($P = 0.0016$). In contrast, differences between slash pine and both the rust-resistant loblolly source and the western loblolly sources were not statistically significant. These trends in SPB mortality between seed sources and species were statistically consistent among blocks according to Tukey's test of nonadditivity, $F (d.f. 1/7) = 0.52$ ($P = 0.4942$) (Tukey 1949).

Table 2. Tests of contrasts among seed source means for SPB mortality.

Contrast	F value	PR > F ¹
Loblolly control vs. loblolly west	18.35	0.0004*** ²
Loblolly resistant vs. loblolly west	3.25	0.0882
Loblolly control vs. slash	13.70	0.0016***
Loblolly resistant vs. slash	1.86	0.1900
Loblolly west vs. slash	0.21	0.6558

¹Probability of a larger value of F given that true seed source means are equal.

²*** = significant at the 0.0102 level.

As reported earlier (Powers and Matthews 1987), rust incidence was significantly higher for both the loblolly and slash commercial controls than for either the western loblolly sources or the rust-resistant eastern sources (Table 1). There was no statistically significant difference between eastern and western sources in the average severity of stem girdling, dbh, or number of trees per acre (Table 1). Again, it is important to note that multivariate analysis failed to show significant partial correlations between rust- or stand-related responses and SPB mortality.

A check of the study area 2 years after the original collection of data found no significant changes in the number of beetle-killed trees.

DISCUSSION

On our test plots in South Carolina, loblolly commercial control trees suffered significantly more SPB-caused mortality than trees from western sources. We believe that ours is the first report of large and highly significant differences in susceptibility to SPB attack among different provenances of a pine species. Our results represent provenance responses to a single population of the beetle. Whether the susceptibility ranking would remain the same with exposure to different beetle populations is not known.

The greater susceptibility of local than western pine sources to SPB attack conflicts with a widely held concept. Many argue that hosts develop tolerance to native pests that have evolved in the same area, and that if the hosts are attacked by these pests damage will be limited. An obvious contrast is the catastrophic result when an exotic pathogen is introduced to a host that has never been exposed, i.e., white pine blister rust, chestnut blight, and gypsy moth.

We first noticed susceptibility of local hosts to local pests in a study with fusiform rust (Powers and Matthews 1980). Loblolly seedlings were grown from seeds collected in six geographic areas across the natural range of the species and were inoculated with rust spores collected from each seed source area. In each series of inoculations, the highest level of rust infection on a specific seed source was caused by the rust spores from its own area.

An additional example from entomology can be found with the fir engraver beetle (Scolytus ventralis LeConte) on white fir (Abies concolor (Gorcl. & Glend.) Lindl. ex Hildebr.). Otrosina and Ferrell (1992) found four times as much mortality in plantations of local provenances (northern California and Oregon) as in plantations containing 39 provenances from throughout most of white fir's range. These results were supported by cage tests where local beetles attack densities were much higher on local than on exotic (Arizona) wood bolts. In addition, the beetles successfully reproduced on the bolts from local trees. Otrosina and Ferrell conclude that local strains of these pests may be better adapted to local tree hosts. Their preliminary work with isozyme analyses and inoculations with the mycangial fungus Trichosporium symbioticum Wright support this hypothesis.

There is no direct evidence in the literature to indicate that beetle populations from various locations differ in their ability to attack pines from different geographic sources. There is, however, some indirect evidence to suggest this possibility. In a laboratory study, Berisford et al. (1990) compared the attractancy to SPB from Texas, Georgia, and Virginia of extracts of billets of loblolly pine infested with beetles from these three locations. Beetles were significantly more attracted ($P < 0.05$) to volatiles from billets infested with beetles from their own locations. Also, loblolly pines from North Carolina were inoculated with isolates of the mycangial fungus Ceratocystiopsis ranaculosus Bridges & Perry of southern pine beetles from North Carolina and Louisiana. The spread of infection was faster for the isolate from North Carolina beetles, indicating a more aggressive fungus attack on native tree hosts. (Cook and Hain, in press). These two studies suggest that local beetle populations are more likely to be successful against native hosts.

Studies have shown differences among widely separated SPB populations in isozymes (Namkoong et al. 1979) and electrophoresis patterns (Anderson et al. 1979). While these results provide no direct evidence that beetle populations from various locations differ in their ability to attack pines from different geographic areas, they support the possibility of differences.

The preliminary findings reported in this paper may provide direction for additional research. These and other seed sources should be studied to determine if the physical and chemical properties of resins are related to differences in susceptibility to SPB attack. Promising seed sources need to be field-tested within and between eastern and western provenances to determine the relative stability of SPB resistance across geographic ranges. Beetle populations need to be carefully monitored to determine the manner of spot initiation and spot growth within isolated plantings of the different seed sources. These kinds of studies would be difficult to design and conduct, but they are necessary to confirm our findings and to locate sources and genotypes that are resistant to SPB attack. Although our study did not reveal any significant relationships among fusiform rust, reduced tree growth, stand stress, and SPB behavior, we feel that a larger, more sensitive study might strengthen our findings and/or provide new insights into such relationships.

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THE EFFECT OF ALTERNATIVE SILVICULTURAL SYSTEMS ON GENETIC DIVERSITY IN DOUGLAS-FIR^{1/}

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Abstract.--The genetic composition (20 allozyme loci) of remaining overstory trees in two-story and group selection plots (6-8 ha) were compared to uncut stands in each of three replications, to assess the impacts of harvesting on genetic diversity in Douglas-fir. In addition, the genetic composition of seven artificial seedling stocks used to reforest the harvested units was examined. Neither harvesting nor artificial reforestation appreciably altered levels of genetic diversity compared to uncut stands.

Key words: Pseudotsuga menziesii var. menziesii (Mirb.) Franco, isozymes, artificial reforestation,

INTRODUCTION

An issue of particular concern today in the practice of forestry is the extent to which management may alter levels of biodiversity in our forests. One important component of biodiversity is genetic variation within species at the population or stand level. This variation is not only vital to adapting species to pest attack and climatic extremes in current environments, but also to their continued evolution in the face of new environments in the future (Ledig 1986). Variation within populations is also the primary source of genetic diversity employed by breeders in tree improvement programs. Despite the importance of genetic variation within populations, little data is available to assess the potential implications of alternative management practices on levels of diversity (Savolainen and Kärkkäinen 1992).

A recent study established by the College of Forestry at Oregon State University, provides an excellent opportunity to address impacts of forest management on biodiversity in a well controlled and replicated experiment. Plant and animal communities under alternative silvicultural regimes are being compared, over time, to those in uncut, mature stands, in each of three replicated blocks. The study summarized in this paper adds genetic composition of the dominant tree species to the higher levels of biodiversity already being

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addressed. The overall objective is to assess the impact of three silvicultural systems (group selection, two-story, and clearcut) on the genetic composition of Douglas-fir [*Pseudotsuga menziesii* var. *menziesii* (Mirb.) Franco] stands when followed by either natural or artificial regeneration. Natural regeneration in harvested areas has not been sufficient as yet to warrant sampling, but data is available on the genetic composition of overstory trees and the artificial stocks used to regenerate the sites. Thus, in this preliminary report, we address the following issues: 1) To what extent does partial harvesting influence the genetic composition of overstories? 2) Do artificial planting stocks differ substantially in genetic composition from uncut stands? 3) To what extent do different sources of planting stock suitable for the same area differ in genetic composition?

Based on the few previous reports of genetic impacts of alternative silvicultural systems (Neale 1985, Savolainen and Kärkkäinen 1992), as well as evidence for high within-population genetic variation and strong gene flow in conifers (Ellstrand 1992, Hamrick et al. 1992), we expected to find little, if any, impact of harvesting in group selection or two-story systems on genetic diversity. Furthermore, unless seed stocks come from very limited numbers of parent trees, artificial regeneration should also possess high levels of diversity (Adams et al. 1992). Given the paucity of actual observations, however, and concerns expressed in professional literature regarding negative genetic impacts of forest management (e.g. Society of American Foresters 1991), the need for additional published data is evident.

METHODS

Study Sites

The study was carried out on the College of Forestry Research Forests, located in the Willamette Valley near Corvallis, Oregon. Distances between the three replicates (blocks) ranged from 3.4 to 7.2 Km. Each block was subdivided into stands of approximately 8 ha, which were subjected to one of the treatments: clearcutting, partial harvesting (group selection and two-story systems), or left uncut. Harvesting was done in two of the blocks in 1990 and in the third block in 1991. In the group selection treatment, all mature trees were removed in 0.20 ha patches scattered throughout the stand. In total, approximately 25% of the stand was harvested. In the two-story treatment, stands were selectively thinned to an average of 18% of the most vigorous trees distributed relatively evenly over the entire area (about 23 trees/ha). With the exception of the few scattered individuals left (and later topped) for wildlife habitat, all trees were removed in the clearcut treatment. Each harvested area was replanted with one or more of a total of seven Douglas-fir seedling stocks originating from two seed zones overlapping the boundaries of the forest. Seed sources were commercial collections in wild stands from two elevational bands (0-152 m, 152-305 m) in seed zone 262 and one elevational band (0-152 m) in zone 252 (Western Tree Seed Council 1966). The seven stocks also differed by the nursery in which they were raised (three) and by stock type (i.e., 1-1 or plug-1). Planting of the harvested areas was begun in the winter of 1990/91 and completed the following winter.

Sampling

One stand of each treatment type (uncut, group selection, two-story, and clearcut) was chosen for sampling in each replicate block. In all stands with overstory trees (i.e., all except clearcuts), a sampling area of approximately 5.0 ha was laid out and twig samples containing dormant buds collected from 120 mature trees located systematically on a grid within this area. In total, 1080 (3 treatments x 3 replicates x 120 trees) overstory trees were sampled. Dormant buds were also collected from 120 seedlings of each of the seven stocks used in planting the harvested areas. Since the seedling stocks were not allocated according to any particular experimental design, and there was no reason to expect samples of the same stock to differ appreciably among planting locations, each stock was sampled in only one area. Immediately after collection, the bud samples from each overstory tree or seedling were sealed in a labeled plastic bag and placed on ice. Subsequently, the samples were kept in a cold (3°C) room until laboratory analysis.

Isozyme analyses

Isozyme analyses were performed on the bud tissues according to procedures described by Adams et al. (1990). In total, 14 enzyme systems (ACO, PGM, PGI, LAP, SDH, GDH, SOD, GOT, G-6PD, F-EST, 6-PGD, IDH, DIA and MDH) and 20 loci were assayed. Genetic diversity statistics [average number of alleles per locus (A), percent of polymorphic loci (P , 99% criterion), and observed (H_o) and expected (H_e) heterozygosities] were estimated for each mature-tree and seedling population sampled. In addition, the extent of genetic differentiation among populations was evaluated by calculating Nei's unbiased genetic distances (Nei 1978) and chi-square statistics for testing heterogeneity of allele frequencies (Workman and Niswander 1970). All calculations were performed using the "BIOSYS" computer program (Swofford and Selander 1989).

RESULTS

Impact of harvesting on the genetic composition of overstory trees

Nineteen of the 20 loci were polymorphic in at least one of the nine stands sampled, and on average, levels of polymorphism in these populations were consistent with earlier studies in this species (Neale 1985, Moran and Adams 1989). Partial harvesting appears to have had little influence on the genetic composition of overstory trees. On average, allele frequencies in the group selection and two-story treatments differed significantly ($P < 0.05$) from the uncut stand in the same replication at 6 of the 20 loci (range 4-9). The largest allele frequency difference at any one locus, however, averaged only 0.07 (range 0.02-0.16). In addition, mean genetic distances between the uncut and group selection stands (0.0017), and between the uncut and two-story stands (0.0023), were small and no greater than the average genetic distance observed among the three uncut stands (0.0027). Estimated levels of genetic diversity also varied little among the three stand types (Figure 1).

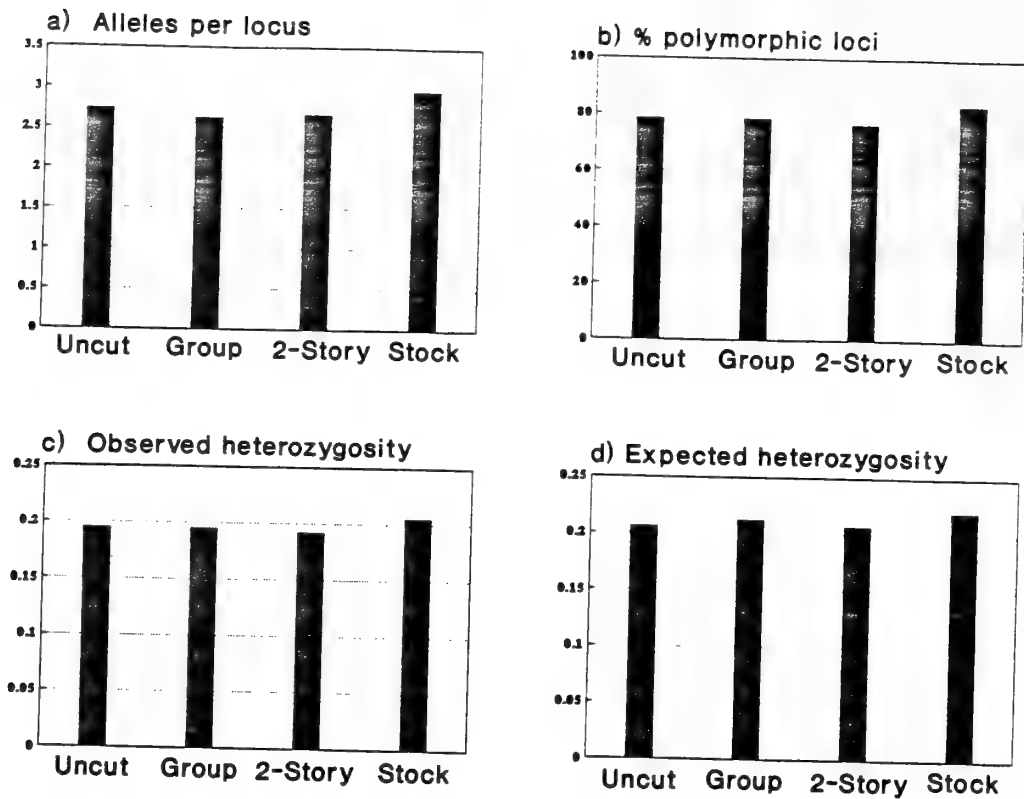


Figure 1. Average number of alleles per locus (a), percent of polymorphic loci (b), observed (c), and expected (d) heterozygosities in Douglas-fir overstory trees and planting stocks.

Genetic composition of planting stocks

Despite the variety of seed sources, nurseries and stock types involved, genetic distances between all pairs of the seven planting stocks never exceeded 0.002 (mean distance 0.0006). The mean genetic distance between the planting stocks and uncut stands in each replication was also small (0.0018, range 0.001-0.004). Levels of genetic diversity in the planting stocks were quite consistent with those found in the uncut stands (Figure 1).

DISCUSSION

The lack of significant changes in levels of genetic diversity due to harvesting confirms results from an earlier investigation in Douglas-fir, where two pairs of shelterwood and adjacent uncut stands were compared (Neale 1985). The numbers of leave trees in the shelterwoods were similar to those remaining after harvesting in the two-story stands in this study. Apparently, reduction in numbers of overstory trees would have to be much greater before losses in allelic diversity due to genetic drift are detectable. Furthermore, even if selection

of overstory trees alters the genotypic mean of the stand for traits selected, the impact on allelic frequencies at individual isozyme loci is expected to be slight, even if the loci directly influence the traits under selection (Lewontin 1984). Thus, both theory and observation confirm that harvesting, per se, has little influence on levels of genetic diversity, as long as numbers of overstory trees are not drastically reduced.

In most cases it is expected that natural regeneration will possess as much or more genic diversity than present in overstory trees. This is because overstory trees not only mate among themselves, but also outcross with trees in neighboring stands. The effective number of parents in overstories, however, could be dramatically reduced in poor seed years, where only a few trees produce pollen and seeds. Therefore, if stands are naturally regenerated in poor seed years, genic diversity in offspring might actually be less than in the overstory.

Levels of genic diversity in artificial stock are primarily a function of the number of parents from which seed is collected. Although the number of parents contributing to commercial seed lots is generally unknown, attempts are made by most forestry organizations in the Pacific Northwest to ensure that wild stand collections include at least 15-30 scattered trees (Adams et al. 1992). In addition, it is recommended that seed orchards contain at least 50 unrelated clones or families. Such numbers seem more than adequate to ensure high levels of genetic variability in planting stock.

The fact that little differentiation was found among the planting stocks at isozyme loci, is not unexpected if relatively large numbers of parents were involved in each stock. In previous studies, genetic distances between coastal Douglas-fir populations, even over fairly wide geographical distances, were shown to be quite small (Merkle and Adams 1987, Li and Adams 1989, Moran and Adams 1989). Thus, isozymes are not very useful for verifying geographical source of parentage (Adams 1983). In this study, it can be assumed that the geographical sources of the planting stocks are appropriate, because the seed originated from the same or adjacent seed zone as the planting locations. Furthermore, there is no evidence that artificial planting stocks contain less genic diversity than found in natural stands.

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PLANT MATERIALS DATABASE DEVELOPMENT AT THE SOUTHERN INSTITUTE OF FOREST GENETICS¹

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Abstract.-- The Southern Institute of Forest Genetics (USDA Forest Service, Gulfport, MS) is working with a large number of plant materials in a variety of research programs. A database has been developed using Borland's PARADOX 4.0™ to track plant materials and record pertinent information about them. Through the databases primary and secondary tables a large amount of information can be efficiently managed. A menu-driven application is being developed to make the data easily accessible.

Keywords: Database management, relational databases, pedigrees, tree breeding, clonal propagation, record keeping.

INTRODUCTION

Since the 1950s, researchers at the Southern Institute of Forest Genetics have selected and maintained a large number of pine genotypes for use in genetic research (Synder et al. 1968). Funding and personnel reductions during the 1970s and 1980s slowed the development of these materials and jeopardized their maintenance at Gulfport and many other labs throughout the South. Because genetic research depends heavily on the availability of diverse and well-characterized genotypes and pedigrees, the Institute has recently expanded its program of collecting and generating these types of research materials. Efforts are primarily focused on producing multi-generation pedigrees for genetic analysis of various traits and preserving diverse materials from native provenances.

To efficiently manage the information associated with the acquired and developed materials, a database using Borland's PARADOX v4.0™ has been created. The purpose of the database is to maintain all pertinent information about the materials in an organized manner and in a form that can be easily accessed by project scientists and technicians as well as non-project personnel. A custom application written in Paradox Application Language™ (PAL) is being prepared for use in performing all the basic functions, such as data protection, data entry and editing, interactive queries (searches and calculations), and report writing. The objectives of this paper are to describe the database's overall structure, including relationships between the tables, and demonstrate its functionality by presenting a detailed example of a complicated query.

DATABASE STRUCTURE

Overview

A database is a series of related (linked) tables (data sets) that are used to store information in an organized way. Tables consist of records (rows) and fields (columns). A table to store height and

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diameter data for a clonal field planting might include the following fields: CLONE, ROW, COLUMN, HEIGHT, and DIAMETER. The data for each tree (ramet) comprises a record, and each field contains a certain type of data. The linkages between tables are designed to minimize the amount of data redundancy, thus maximizing storage and retrieval efficiency. For example, to keep all the data for a clone in one table would be cumbersome, especially if different kinds of data were being kept. A "normalized" database would contain data for the clone in several tables, with each table containing different kinds of information (Borland International, 1992). Thus, when searching (querying) for specific information about the clone (e.g., species, provenance, pedigree, rooting data, field growth data, etc.), only the pertinent tables need to be accessed. During the query, the database program "links" tables through fields included in both tables to properly associate (merge) the corresponding records and return the information requested.

Primary tables. The central table of our database is the *clone*³ table. Information specific to the clone (genotype) is entered into the *clone* table. Each ramet of a clone is entered into the *ramet* table along with a code indicating its physical location. A table for each possible location lists the included ramets and other information about the ramets at that location. Pedigree information is stored in the *pedigree* table and provenance information in the *provenance* table. Because clones are part of active or closed experiments, critical information on these experiments is stored in the *experiment* table.

Secondary tables. The key to all the possible physical locations for a ramet is given in the *location* table. A series of tables (*ab*, *cb*, *co*, and *field*) contain information on these locations. Pollination records and pollen and seed storage records are kept in the *flowers*, *pollen*, and *seed* tables, respectively. A large group of tables contains data (such as rooting or field growth measurements) on ramets or seedlings that are involved in various experiments. Seedlings selected from these will be given a clone number and then vegetatively propagated for use in future research. The names of these tables are usually prefixed with the experiment code such as *SD18-27*, *SD3*, or *SD4*.

Clones and Ramets

Information unique to a clone is maintained in the *clone* table. *Clone* includes the following fields: CLONE, ALIAS, SPECIES, EXPERIMENT, and ENTRY. CLONE is used to record the clone number. A clone number is assigned to a plant when it is vegetatively propagated or if it is part of another clone's pedigree. ALIAS is a record of past identification codes. SPECIES is a two-letter to four-letter code based on the Latin specific name. EXPERIMENT is a two-letter code for the experiment in which the clone was originally entered. ENTRY is generally synonymous with family. Entries within an experiment are numbered "1" to "n". EXPERIMENT+ENTRY defines a specific family (all clones with the same EXPERIMENT and ENTRY are full or half siblings), with the exception of wild trees (trees of unknown parentage). Wild trees are entered as experiment "ZZ". The entry here refers to the provenance of the tree, not the family. Thus, two trees of entry "2" in experiment "ZZ" are wild trees from the same provenance.

The *ramet* table contains information specific to a particular ramet of a clone. *Ramet* includes the following fields: CLONE, RAMET, and LOCATION. The seedling ramet (ortet) is always assigned RAMET "0". A key to location codes is found in the *location* table. Some examples of locations are accelerated breeding greenhouse, clone bank, cutting orchard, or dead. A table for each location (e.g., *AB*,

³ To clarify references between tables, fields, and values, the following conventions are used throughout this paper: table names are italicized (*clone*); field names are in all capitals (CLONE); and values are in double quotes (clone "10").

CB, CO) gives the specific information about each ramet, including its coordinates within the location. Figure 1 shows the *clone* and *ramet* tables.

Image Undo ValCheck DO-IT! Cancel						
Clone						
CLONE	Clone	Alias	Species	Experiment	Entry	
1	10		EL	SD	25	
2	91		EL	SD	23	
3	463	25-2	EL	AA	25	
4	468	23-1	EL	AA	23	
5	1002	8-7	EL	ZZ	1	
6	1003	9-2	EL	ZZ	1	
7	1005	18-62	EL	ZZ	1	

[J] Ramet [↑]			
RAMET	Clone	Ramet	Location code
1	10	0	GG
2	91	0	GG
3	463	0	FI
4	463	A	AB
5	463	B	AB
6	468	0	FI
7	468	A	AB
8	1002		CB
9	1005		CB

1 of 10

F1 Help F7 Form Edit

Figure 1. An image of a computer screen displaying the *clone* and *ramet* tables. *Clone* contains information unique to the genotype. *Ramet* contains information on individual ramets. Table fields are explained in the text.

Pedigrees and Provenances

As explained in the previous section, each EXPERIMENT+ENTRY combination defines a family or a provenance. The *pedigree* table records all pedigree information for each clone and family. Its four fields are EXPERIMENT, ENTRY, FEMALE (clone number of female parent), and MALE (clone number of male parent). Wild trees are assigned to experiment "ZZ". Provenance information is stored in the *provenance* table. Trees of unknown provenance are assigned entry "0". Known provenances are coded "1" to "n". Figure 2 displays a sample of the *pedigree* and *provenance* tables.

Linking Clones, Pedigrees, and Provenances

One of the most frequently performed database tasks is researching a clone's pedigree or provenance. The link between a clone and its parents is EXPERIMENT+ENTRY. Figures 1 and 2 show views of the *clone*, *ramet*, *pedigree*, and *provenance* tables, which may be consulted to follow the examples given. Assume a user wishes to find out about clone "10" and find all of its full siblings. Clone "10" the first clone listed in the *clone* table, was originally in experiment "SD" and entry "25". Experiment "SD" and entry "25" are found in record 8 of the *pedigree* table. The parents of clone "10" are clone "1002" as female and clone "1005" as male. To further examine the pedigree, the parents of clones "1002" and "1005" can be found-- first by finding their EXPERIMENT and ENTRY in the *clone*

table and second by linking to the *pedigree* table. In the *pedigree* table, clone "1002" can be found in record 5 and clone "1005" in record 7. Both trees originated in experiment "ZZ" and entry "1" informing the user that both are wild trees and of the same provenance. To identify the provenance, the *provenance* table is searched for entry "1". Record 2 of the *provenance* table shows entry "1" to be Harrison County, MS. Thus, clone "10" is a control-cross offspring of two wild trees both from Harrison County, MS.

View Ask Report Create Modify Image Forms Tools Scripts Exit					
Pedigree					
PEDIGREE	Expt	Entry	Female	Male	
1	AA	23	1002	1003	
2	AA	25	1002	1005	
3	AD	19	1266	1271	
4	AD	25	1258	1277	
5	AD	7	1260	1276	
6	SD	22	1002	1002	
7	SD	23	1002	1003	
8	SD	25	1002	1005	

Provenan				Provenance
PROVENAN	Expt	Entry		
1	ZZ	1	Harrison co. MS	
2	ZZ	0	UNKNOWN	

1 of 2

F1 Help F7 Form Alt-F9 CoEdit Main

Figure 2. *Pedigree* and *provenance* tables. *Pedigree* contains parent information for trees of known parentage. *Provenance* contains provenance information for wild trees. Table fields are explained in the text.

To find all the full siblings of clone "10" the *pedigree* table is first searched for all records having "1002" and "1005" as parents (either female "1002" and male "1005" or female "1005" and male "1002"). Record 2, experiment "AA" and entry "25", and record 8, experiment "SD" and entry "25", are the only matches. Next, to find the clone numbers of the full siblings, the *clone* table is searched for clones matching experiment "AA" and entry "25" or experiment "SD" and entry "25". Clone 10 (the original clone of interest) and clone "463" are the only matches. Finally, to locate all ramets of clones "463" and "10" the *ramet* table is searched. Four ramets are found-- clone "10" ramet "0" (i.e., the original stock plant of clone "10") at location "GG" and clone "463" ramets "0", "A", and "B" at locations "FI", "AB", and "AB", respectively.

Locations and Associated Tables

The *location* table is a key to the location codes found in the *ramet* table. It includes a brief description of the location, the names of associated tables, and the name of a technician or scientist to contact for further information. To continue the example from the previous section, clone "463" ramets "A" and "B" are found at location "AB". The location table (Figure 3) shows that "AB" refers to the

accelerated breeding greenhouse, the associated table is *ab*, and the contact person is "Mary Mason". By searching the *ab* table (see Figure 3) for clone "463" ramets "A" and "B", information on the greenhouse location (block), the potting media (pot mix), and the grafting rootstock of ramets "A" and "B" can be found (without bothering Mary). Most locations have an associated table. The location tables' first two fields are always CLONE and RAMET. The remaining fields contain data pertinent to that location. For example, the fields for the *cb* (clone bank) table are: CLONE, RAMET, GC (propagation information), ROW, COLUMN, YEAR_IN, and ROOT_STOCK. Currently, location tables are available for the accelerated breeding greenhouse, the clone bank, the potted and field cutting orchards, and several field plantings.

View Ask Report Create Modify Image Forms Tools Scripts Exit									
Ab									
AB	Clone	RAMET	GC	BLOCK	YR IN	POT MIX	ROOT STOCK		
26	462	B	OG	5	1989	1			
27	462	C	OG	15	1990	2			
28	462	D	OG	1	1989	1			
29	463	A	OG	1	1989	1	W-1-20		
30	463	B	OG	5	1989	1	W-1-20		
31	464	A	OG	9	1990	2	8-7		
32	466	A	OG	9	1990	2	8-7		
33	467	A	OG	7	1990	2	W-1-20		

Location				
LOCATION	Location code	Location name	Assoc tables	Contact
1	AB	accelerated breeding ghs	AB	Mary Mason
2	CB	clone bank	CB	Mary Mason
3	GG	Gulfport greenhouses		Tom Caldwell
4	HO	hedging orchard		Tom Caldwell
5	FI	Field planting	FIELD	
6	HG	HEF Greenhouses		

1 of 6

F1 Help F7 Form Alt-F9 CoEdit Main

Figure 3. *AB* and *location* tables. Information on trees in the accelerated breeding facility is found in *ab*. *Location* contains information about possible locations of ramets. Table fields are explained in the text.

Other Tables

Records on pollinations completed and pollen and seed storage are also included in the database. The *flowers* table records information from the field pollination records including: year, female clone, female ramet, tag number, number of conelets pollinated, average conelet maturity, date of pollination, male clone, male ramet, and pollen age. The table is updated with 6-month cone counts, cone collection counts, and pollination or cone collection remarks. Seed lots are entered into the *seed* table, containing information such as year collected, female and male parents (clone and ramet), 100-seed weight, number of seeds, and storage location. Open-pollinated and pollen mix seed lots are assigned to male parent clone "0". A separate table, *Male_0*, records information on specific pollen mixes (open-pollinated seed lots are assigned pollen mix "0").

The final part of the database is a group of tables containing information on experiments. *Experiment* is a master table of experiments similar to the *location* table. It gives a key to experiment codes, a brief description of the experiment, and a contact person. Tables for individual experiments generally take one of three forms depending on the propagation history of the trees. The first form contains ramets of established clones, the second seedlings, and the third a combination of ramets and seedlings. All of these tables include pertinent experimental data such as cutting production and rooting percentage for a rooting experiment, or planting location (row, column) and measurements for a field planting. Pedigrees for clones are accessed by linking to CLONE in the *clone* table to find EXPERIMENT and ENTRY information, and then linking to *pedigree*. Seedlings are given a plant number within the experiment. EXPERIMENT and ENTRY information for each plant (seedling) is recorded in the data table for the experiment. The parent information is recorded in the *pedigree* table. Upon selection for clonal propagation or breeding work, seedlings are assigned a clone number, the seedling is assigned RAMET = 0, and the plant number is recorded as ALIAS. Some of these tables of the second and third form may be "hidden" from the user because they are not based entirely on clone number. An indication of their existence may include one of the following: an entry in the *pedigree* table to which no clones belong, an entry in the *experiment* table, or a group of small seed lots (remnant seed) in the *seed* table.

USING THE DATABASE

Query-By-Example

To understand how to use the database, it is important to know about several features of relational databases. Tables can be viewed, edited, or queried. When in view or edit mode, the zoom feature can be used. Zoom searches the table for the first record in a chosen field matching an input value and advances the cursor to that record. It is generally quick to use zoom to locate records in one table pertaining to one or two clones. To obtain information involving a larger number of records or from more than one table, it is generally faster to construct a query. Paradox uses "query-by-example," which involves filling out a "query form." Fields to be included in the "answer" table are "checked," and selection criteria are typed into the appropriate fields. Links between tables are established by typing the same "example element" (series of characters) into the common fields in each table involved in the query. The example element serves only to identify the common fields to Paradox, so the choice of characters is arbitrary.

The best way to illustrate how to use the database is to return to the example begun in Linking Clones, Pedigrees, and Provenances. To begin to find the pedigree and full siblings of clone "10" the user would view the *clone* table to find clone "10" and note its experiment and entry information ("SD" and "25"). Next, the user would browse the *pedigree* table to find the parents of "SD"+"25" (clones "1002" and "1005"). Knowing the parent clone numbers, the user can return to the *clone* table to find their experiment and entry information, then browse *provenance* to find their provenance.

Since up to this point only one or two clones are being considered, it is efficient to simply browse the tables. To find all the full siblings of clone "10" it is better to use a query. To perform the query, the user would fill in the query form as shown in Figure 4. The first line of the form specifies a search for the cross "1002" x "1005", and the second for the cross "1005" x "1002". The answer table returned for this query is also shown in Figure 4. A more complex query involving a link between *clone* and *ramet* will return all ramets of the clones of interest in one step. Figure 5 shows the necessary query and the answer table it returns. The example element "abc" allows Paradox to find the ramets of the desired

clones. The answer table can be renamed and saved for future reference or printed out under a standard or a custom report format.

The screenshot shows a menu-driven interface with the following menu bar: View Ask Report Create Modify Image Forms Tools Scripts Exit.

The main window is titled "Query Pedigree" and contains a form with the following fields:

- PEDIGREE**: A dropdown menu showing "1 of 2".
- Expt**: A text field containing "AA".
- Entry**: A text field containing "25".
- Female**: A text field containing "1002".
- Male**: A text field containing "1005".

Below the form is an "ANSWER" table with the following data:

ANSWER	Expt	Entry	Female	Male
1	AA	25	1002	1005
2	SD	25	1002	1005

At the bottom of the window is a status bar with the following text: F1 Help F6 Alt-F6 Ctrl-F6 F2 DO-IT! Main

Figure 4. A query to return all clones that are full siblings of clone "10", a control-cross offspring of clones' "1002" and "1005". The first line of the query returns entries in the cross "1002" x "1005". The second line returns cross "1005" x "1002". The entries of interest are shown in the *answer* table.

Custom Application

A custom application is being developed to facilitate access to the database for those who are unfamiliar with it. A custom application offers several advantages over interactive use. First, it provides better security against accidental loss or corruption of data. Second, it allows tasks to be programmed so that a user can get information without having to know the details of the database program. For example, a pedigree can be researched without the user having to fill out a query form with complex links between tables. The application can be menu-driven so that a user can select a task from the menu, fill in a pop-up "dialogue box" to provide the application with needed details to complete the task, and view the desired information on a form with supplemental or explanatory text.

Application modules for use in a complete custom application for managing the database are currently being developed. The completed application should allow a user to see an overview of the database, enter data into various tables, ask a wide range of questions about the data (searches and calculations), and receive printed reports detailing the results of the queries. Extensive interactive use is required to identify the queries most often constructed and thus most necessary for inclusion in the custom application. Anticipated menu-driven queries include the following: constructing complete multi-generation pedigrees; searching for clones, ramets, seed, and pollen lots, etc.; and calculating numbers of clones per family, ramets per clone, and number of seeds per cross, etc.

View		Ask	Report	Create	Modify	Image	Forms	Tools	Scripts	Exit																																										
CLONE		Clone		Query Clone		Species		Experimen																																												
		abc, 463 or 10																																																		
RAMET		Clone		Query Ramet		Location code																																														
		abc																																																		
<table border="1"> <thead> <tr> <th colspan="6">ANSWER</th> </tr> <tr> <th>ANSWER</th> <th>Clone</th> <th>Experiment</th> <th>Entry</th> <th>Ramet</th> <th>Location code</th> </tr> </thead> <tbody> <tr> <td>1</td> <td>10</td> <td>SD</td> <td>25</td> <td>0</td> <td>GG</td> </tr> <tr> <td>2</td> <td>463</td> <td>AA</td> <td>25</td> <td>0</td> <td>FI</td> </tr> <tr> <td>3</td> <td>463</td> <td>AA</td> <td>25</td> <td>A</td> <td>AB</td> </tr> <tr> <td>4</td> <td>463</td> <td>AA</td> <td>25</td> <td>B</td> <td>AB</td> </tr> <tr> <td>5</td> <td>463</td> <td>AA</td> <td>25</td> <td>C</td> <td>AB</td> </tr> </tbody> </table>											ANSWER						ANSWER	Clone	Experiment	Entry	Ramet	Location code	1	10	SD	25	0	GG	2	463	AA	25	0	FI	3	463	AA	25	A	AB	4	463	AA	25	B	AB	5	463	AA	25	C	AB
ANSWER																																																				
ANSWER	Clone	Experiment	Entry	Ramet	Location code																																															
1	10	SD	25	0	GG																																															
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4	463	AA	25	B	AB																																															
5	463	AA	25	C	AB																																															
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Figure 5. Query to find all ramets of clones "10" and "463", and their locations. "abc" is an example element linking the CLONE field in the two tables *clone* and *ramet*. "463" and "10" are selection criteria for the CLONE field. The *answer* table shows the ramets of interest.

SUMMARY

A database has been developed to track plant materials maintained and developed at the Southern Institute of Forest Genetics. Through several primary tables, the database maintains clonal identification, pedigrees, provenances, location of ramets, and pertinent details about ramets at each location. Secondary tables contain pollination and seed records and experimental data. The database allows reliable, quick, and easy access to a large amount of information, resulting in more research time and less hard copy search time. A menu-driven custom application is being developed to ensure these advantages are not lost for those who only occasionally use the database.

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Dendrome, A GENOME DATABASE FOR FOREST TREES

B.K. Sherman and D.B. Neale^{1/}

Abstract.-- The Dendrome project is creating an archive of genome maps, analysis tools, and data visualization technologies of particular interest to forest molecular geneticists. Services are provided through the Internet worldwide computer network. These services afford access to genome databases, images, announcements, software and expertise. Connections to technically related archives and services are supported.

Keywords: Pinus taeda, database, bioinformatics

INTRODUCTION

The Dendrome project is an attempt to construct a framework for the acquisition, storage and retrieval of genome data of forest trees. Dendrome databases are designed to be research tools for forest geneticists and other forest biologists. The first genome database offered is of loblolly pine (Pinus taeda L.). This software is APtDB (A P. taeda Database) and it includes the maps and data resulting from mapping projects at the Institute of Forest Genetics. This database offers a sophisticated user interface and allows graphical display of both genetic and physical maps. It is intended that maps from other laboratories will be brought into congruence and the data merged into a consensus map. Certain other services are in place and can be accessed via computer networks from locations around the world. These include retrieval of images of autoradiograms, textual representations of data in APtDB, announcements, protocols and interconnection with related services.

The genome maps represent chromosomal locations of genes. The databases will also include information associated with genes such as nucleotide and amino acid sequences, patterns of gene expression, links to metabolic pathways, and sizes of gene families. Information of this type could be used by molecular biologists and physiologists. There will be information on the amount and type of allelic variability of mapped genes. This information could be used by population and evolutionary geneticists. There will be information on map position of quantitative trait loci for important traits. Quantitative

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geneticists and breeders might use this information for experimental purposes or even practicing marker-assisted breeding.

The Dendrome project is funded by the United States Department of Agriculture Agricultural Research Service Office of Plant Genome. It is one component of a three to five year collaborative project to construct prototypes for conifers and other species: Arabidopsis thaliana, maize, soybeans, and wheat. Data collected by the various projects will be cross-referenced into a centralized Plant Genome Database to be administered by the National Agricultural Library.

HARDWARE AND NETWORKING

The primary computer for Dendrome is a Sun Microsystems SPARCstation 2. This computer has 64 megabytes of RAM, and five gigabytes of storage on hard disk drives in addition to tertiary storage on tape media and optical disks. This machine acts as the main server for resources described below.

A LAN (Local Area Network) provides connections to various smaller computers, printers and input devices. A router on the LAN provides a connection to BARRNet (Bay Area Regional Research Network) and the Internet. A Forest Service computer on the LAN gives a connection to the Forest Service wide area network. For a complete discussion of the relationship of the Internet to other computer and telecommunication networks, see Quarterman (1990).

Image acquisition is done with a Cohu charge-coupled device camera, Epix framegrabber, and an Intel-architecture computer. The image data is transferred to the Sun workstation for analysis, manipulation and export. A Umax scanner is available for high resolution color images.

SOFTWARE

The primary repository of genome data is APtDB which employs the ACEDB software (Durbin 1990). Genome data is characterized by deep knowledge of a few items and little about most. This model is not tractable by conventional relational database systems. ACEDB is an attempt to use an object-oriented approach to encode both genetic and physical data. It uses graphical displays, extensive cross-referencing and both a keyboard and mouse to allow easy navigation and visualization of the data and their relationships. Images of the autoradiograms that were used to create the genetic map of loblolly pine are indexed into APtDB.

The X Window System provides a platform-independent mechanism for giving users access to a common graphical interface. Using X, APtDB, which at present runs only on Unix platforms, can be running on the Sun workstation yet have the window displays and user input occur on other machines on the network, an Apple Macintosh, say, or an Intel 80486 computer running Microsoft Windows.

Remote retrieval of data from Dendrome does not require sophisticated computers. A terminal, a modem and a telephone line are sufficient to use two main services: gopher and WAIS. These protocols allow purely textual interaction with the Dendrome databases, but still offer a powerful and concise mechanism for information retrieval.

Gopher is an interactive menu-based tool that allows one to navigate the Internet searching for services. Moving from machine to machine is accomplished without passwords or knowledge of machine names or locations. Machine architectural differences are hidden by the protocol. Gopher can offer access to Wide Area Information Servers (WAIS). WAIS allows huge collections of textual data to be indexed by every word in the data. The index is very large, often larger than the data but once the index is built, novel searches can be accomplished extremely quickly. Both the index and the data are made available to WAIS clients by WAIS servers. Dendrome offers several WAIS indexed collections. Information located via gopher and wais can be stored in a file on the user's local computer or electronically mailed to a colleague. For a more complete description of these protocols, see Krol (1992).

Scientists at the Institute of Forest Genetics are experimenting with various genetic linkage analysis software including: GMendel, Mapmaker, Crimap, and Joinmap. Various image manipulation software is being used and evaluated including: xv, pbmplus and HIPS.

CONCLUSION

Contemporary genetics is characterized by exponentially increasing quantities of data. The quantity and structure of genome data imply the collaborative use of computers and high speed communication networks. Making biological inferences based on that data requires ways of visualizing the data at various levels of detail. Efforts of individual researchers can be amplified if data can be easily shared between laboratories. Many resources are already available to biologists only via the Internet (Smith 1993). The Dendrome project is an attempt to make such technology available for forest biology investigations. The ultimate measure of Dendrome will be whether or not researchers use it.

HOW TO CONTACT Dendrome

Internet electronic mail: **Dendrome@s27w007.pswfs.gov**

Gopher Server: **s27w007.pswfs.gov port 70**

The IP address of s27w007.pswfs.gov is **192.131.1.21**

Surface mail: **Dendrome Project**
Institute of Forest Genetics
P.O. Box 245
Berkeley, CA 94701

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RELATIONSHIPS BETWEEN MONOTERPENE COMPOSITION AND FUSIFORM RUST RESISTANCE

M. Michelozzi¹, A.E. Squillace², and W.J. Lowe³

Abstract. Previous reports, dealing with the relationships between monoterpene composition and fusiform rust resistance in loblolly and slash pines, suggest that trees having relatively high β -phellandrene are more often resistant to the disease than low β -phellandrene trees. Although causes of the relationship are not clear, the results suggest that monoterpene composition be utilized as an early selection aid by culling low β -phellandrene phenotypes.

Keywords: Loblolly pine, slash pine, Pinus taeda, Pinus elliottii, monoterpenes, Cronartium quercuum f. sp. fusiforme

INTRODUCTION

Development of strains of trees strictly for resistance to fusiform rust [Cronartium quercuum (Berk.) Miyabe ex Shirai f. sp. fusiforme] would be relatively easy. Selection of rust-free trees in highly infected stands can be effective. But, because most tree breeders are interested in superiority of other traits along with rust resistance, the task is more complicated. Usually selections are made in sexually mature stands with great attention paid to superiority in traits other than rust resistance. Although infected trees are avoided, more effort is required to incorporate rust resistance. Usually seeds are collected from preliminary selections and these are tested for rust resistance by artificial inoculation in greenhouse tests (Anderson 1982) or in short-term progeny tests. Several early studies suggested the possibility of using monoterpene composition as an aid in selecting trees for rust resistance. Results of these studies along with two more recent and comprehensive tests, are briefly reviewed. Implications and practical applications are then discussed.

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LOBLOLLY PINE STUDIES

Hare (1970) analyzed monoterpenes of stem xylem oleoresin in seed source plantations of loblolly pine (*P. taeda* L.). Trees from relatively rust resistance sources were found to contain more β -pinene and limonene than susceptible sources.

Rockwood (1973) found no correlations between rust resistance and monoterpene composition of stem xylem oleoresin in loblolly pines. But using cortical oleoresin, he found that relatively resistant trees tended to contain less β -phellandrene than susceptible trees. This contrasted with subsequent reports and we should note that sampling here was restricted to trees native to southwest Georgia.

Squillace and Wells (1981) studied cortical monoterpenes of loblolly pines grown from seed collected over the entire range of the species. Although results were not conclusive, trees having relatively low myrcene, high limonene, and high β -phellandrene tended to be more resistant to fusiform rust than other trees (Table 1).

Table 1. Differences in frequencies of rust-free and rust-infected loblolly pines classified as having (1) low myrcene-high limonene-high β -phellandrene (mLP trees) or (2) all other monoterpene phenotypes (adapted from Squillace and Wells 1981).

	Rust-free trees (no.)	Rust-infected trees (no.)	Percent rust-free (percent)
"mLP" trees	297	101	74.6
Other trees	1050	680	60.7
Totals	1347	781	

Contingency test: X^2 , adjusted for continuity, with 1 DF = 26.43, highly significant.

Squillace et al. (1984) studied cortical monoterpene composition of juvenile loblolly pine families having varying degrees of resistance to rust in three plantations. Trees classified as having relatively high β -pinene, low myrcene, high limonene, and high β -phellandrene (Squillace et al. 1980) were usually more resistant than other trees under three measures of degree infection (Table 2).

Table 2. Comparison of three measures of fusiform rust infection in BmLP¹ trees vs. other trees, in three plantations of Georgia.²

	Plantation No. 301 (227 trees)	Plantation No. 302 (97 trees)	Plantation No. 303 (594 trees)
	Percent Infected		
BmLP trees	31	43	12
Other trees	46	43	17
	Average Number Infections Per Tree		
BmLP trees	0.5	1.7	0.1
Other trees	1.9	2.8	1.2
	Average Number Infections Per Infected Tree		
BmLP trees	1.5	4.0	1.0
Other trees	4.0	5.8	1.3

¹ Trees having relatively high β -pinene, low myrcene, high limonene, and high β -phellandrene.

² Data from Squillace et al. (1984) in a study sponsored by the Georgia Forestry Commission.

Using data obtained by Squillace and Wells (1981), Squillace et al. (1985) showed that natural selection in loblolly pines in western and central portions of the species range tends to favor trees having high β -pinene, low myrcene, high limonene, high β -phellandrene. This phenotype (BmLP) presently comprises a large proportion of western populations which are relatively resistant to fusiform rust. Although the phenotype is rather infrequent in central populations it is presumably increasing with each generation. Curiously, loblolly pines in northeastern regions also tend to be rust resistant, but unlike western trees, bMIP trees were found to be favored by natural selection. In 1992 we sampled cortical monoterpenes in 38 rust resistant and 35 susceptible loblolly pine clones. Clones classified as having high β -phellandrene were significantly more frequent in the resistant group than in the susceptible group (Table 3). Although different individual monoterpene types were too few for reliable comparisons, the BmLP clones were most often resistant while BMIP clones were most often susceptible.

SLASH PINE STUDIES

Rockwood (1974) sampled cortical monoterpenes in relatively resistant and relatively susceptible slash pine (*P. elliotii* Engelm. var. *elliotii*) clones, families, and individuals. Although results were somewhat variable, resistant trees usually contained more β -phellandrene than susceptible ones. Results were clear enough to suggest using monoterpene composition as an aid in selecting rust resistant trees in breeding programs.

Table 3. Numbers of resistant and susceptible loblolly pine clones classified by relative content of β -phellandrene in cortical oleoresin. Data from Michelozzi et al. (1992).

β -phellandrene class (percent)	No. clones resistant	No. clones susceptible	Percent resistant
Low β -phellandrene			
0 to 3	6	17	26.1
High β -phellandrene			
5 to 15	25	13	65.8
16 to 30	7	5	58.4

Results of contingency tests (adjusted for continuity): Low vs. high classes, X^2 with 1 df = 7.62, highly significant. Between the two high classes, X^2 with 1 df = 0.02, nonsignificant.

Michelozzi et al. (1990) studied cortical monoterpenes in 85 north Florida slash pine clones progeny-tested for rust resistance. Fifty-seven percent of the trees having relatively high β -phellandrene were resistant, while only 15 percent of the low β -phellandrene trees were resistant (Table 4). Among trees classified as having relatively high β -phellandrene, there was no correlation with rust resistance. Thus, increasing β -phellandrene beyond a critical level seemed to have no effect on resistance. Although high limonene trees are rare in north Florida, four of the 73 trees tested contained high limonene. Three of these were resistant, which tends to agree with earlier work, but more tests would be required to prove a relationship of limonene content with rust incidence.

Table 4. Number of resistant and susceptible slash pine clones classified by relative content of β -phellandrene in cortical oleoresin. Data are from Michelozzi et al. (1990).

β -phellandrene class (percent)	No. clones resistant	No. clones susceptible	Percent resistant
Low β -phellandrene			
0 to 3	2	11	15.4
High β -phellandrene			
5 to 15	12	7	63.2
16 to 25	15	14	51.7
26 to 57	14	10	58.5
Totals	41	31	56.9

Results of contingency tests: Low vs. high classes, X^2 adjusted for continuity, with 1 df = 6.04, significant at the 0.05 level. Among high classes, X^2 adjusted for continuity, with 2 df's = 0.64 nonsignificant.

Forty-six additional slash pine clones were sampled in the 1992 study by Michelozzi et al. The results showed that clones with low β -phellandrene tend to be more susceptible to fusiform rust than trees having β -phellandrene as found in previous data (Table 5). Unfortunately, no BmLP or BMlp types occurred among these clones.

IMPLICATIONS

Although results of the various studies are somewhat variable, high β -phellandrene trees were more frequently resistant to fusiform rust than low β -phellandrene trees. High limonene trees were also more often resistant than low limonene trees, but more tests are required to prove this.

Reasons for the relationships are not clear. Rockwood (1974) suggested that toxicity of the chemicals does not appear to be important. The lack of an increase in resistance with increasing content of β -phellandrene beyond a critical level also suggests the chemical itself is not the cause. Terpenes may act in association with other important factors of resistance. Perhaps the presence of the gene (or genes) that cause high β -phellandrene may be indicative of resistance through linkage or

Table 5. Numbers of resistant and susceptible slash pines classified by relative content of β -phellandrene. Data from Michelozzi et al. (1992).

β -phellandrene class (percent)	No. clones resistant	No. clones susceptible	Percent resistant
Low β -phellandrene			
0 to 3	5	14	26.3
High β -phellandrene			
5 to 10	<u>18</u>	<u>9</u>	<u>66.7</u>
Totals	23	23	50.0

Contingency test: X^2 adjusted for continuity, with 1 df = 5.74, significant at the 0.05 level.

correlations with other traits more directly related to resistance. Hare and Switzer (1969) suggested that introgression with shortleaf pine (*P. echinata* Mill.) may explain rust resistance in western loblolly pines. Likewise, Saylor and Kang (1973) suggest that resistance of loblolly pine in the northeastern region may be due to introgression with pond pine (*P. serotina* Michx.). Unfortunately, cortical monoterpenes of these pine species have not been well studied. Results also suggest that particular combinations are more indicative of resistance than a certain monoterpene alone, and this would result in more powerful predictors of field performance. More comprehensive tests would be desirable.

APPLICATION

We believe that the relationship between relative content of β -phellandrene and fusiform rust resistance is strong enough to use as an aid in developing rust resistance strains of loblolly and slash pines. A desirable procedure would be to determine monoterpene composition of candidate trees prior to progeny testing followed by culling trees having low β -phellandrene. Progeny testing, possibly including short-term artificial inoculation tests for rust resistance, would still be required. But sampling and analyzing for monoterpene composition can be done rapidly at relatively low cost and would greatly decrease the cost of field progeny testing. In view of Rockwood's (1973) findings, the procedure may not be effective with loblolly pine in southwest Georgia.

ACKNOWLEDGMENTS

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SESSION 7

Breeding and Progeny Testing

Family x Environment Interaction for Sweep in Local and Nonlocal Seed Sources of Loblolly Pine.

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Abstract -- Eight tests were planted in Mississippi, Arkansas and Oklahoma across diverse physiographical areas with each test containing five loblolly pine (*Pinus taeda* L.) seed sources: coastal North Carolina, piedmont North Carolina, northern Mississippi/Alabama, central Mississippi/Alabama and Arkansas/Oklahoma. Each seed source consists of the best eight open-pollinated families for volume available from first generation seed orchards. The fastest growing seed source across sites at age eight was coastal North Carolina (28.24 dm³/tree) and the slowest was Arkansas/Oklahoma (22.60 dm³/tree). Piedmont North Carolina had less sweep (2.80 cm) than all other seed sources while the central Mississippi/Alabama seed source was the most crooked (3.13 cm). There was considerably more G x E for sweep among seed sources than families within seed sources. Conversely, for volume there was slightly less G x E at the seed source level than for families within seed sources for volume per tree. The more unstable seed sources for sweep were the coastal North Carolina source which dropped significantly in rank at two Mississippi sites and the central Mississippi/Alabama source which slightly dropped rank when planted northwest of its origin. There was no correlation among families for sweep and volume.

Keywords: Bole straightness, genetic stability, *Pinus taeda* L.

INTRODUCTION

Stem straightness has been an important trait in conifer breeding programs worldwide, especially in breeding programs which focus on improved sawlog grade. An ocular score based on an absolute rather than relative scale has proven the most beneficial (Miller 1975; Bannister 1979; Mullin et al. 1969). With the absolute score, grades assigned to each tree are based on the actual amount of defect observed; the score value is not relative to other trees at the location. There are three primary benefits of using an absolute value for stem straightness rather than a relative score: 1) maladaptability and other types of genotype x environment interaction (GxE) can be readily detected across locations, 2) score values can be translated into financial values at a

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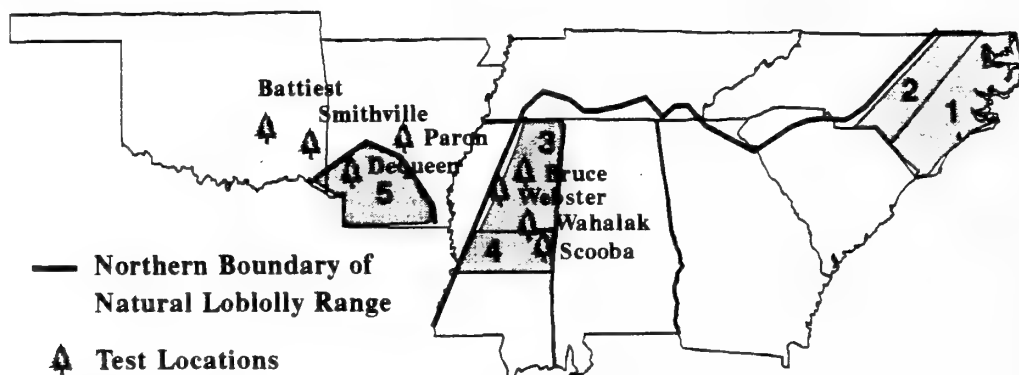
mill conversion facility and 3) selection for straight trees is more efficient across locations. In Weyerhaeuser's loblolly pine breeding program, a six-point absolute ocular score is used to measure sweep in the first log (Williams and Lambeth 1989).

Maladaptability has been observed in sweep measured in southerly seed sources of loblolly pine (*Pinus taeda* L) planted at a single Arkansas location. However, without multiple planting locations of the same seed sources, the study could not be used to ascertain whether significant G x E existed for sweep (Williams and Lambeth 1989). In other studies, stem straightness showed less GxE than diameter in radiata pine (*Pinus radiata* D. Don.) in Australia (Pederick 1986). Stem straightness in loblolly pine (*Pinus taeda* L.) from the "Lost Pines" area in Texas showed no significant GxE although GxE was strong for height and volume per tree (van Buijtenen 1978). However, neither study used a fixed ocular score based on sweep. Due to observed sweep problems with some non-local seed sources in Arkansas and Oklahoma (Lambeth, et. al. 1984), further study is needed to see whether local and non-local seed sources and families are stable (low G x E) for sweep ranking across a large number of sites.

This study examines the magnitude of genotype x environment interaction for sweep in the first log as compared to volume per tree at age eight years. Sweep and growth were measured in tests planted in Mississippi, Arkansas and Oklahoma which had open-pollinated families within five loblolly pine (*Pinus taeda* L) seed sources of both local and non-local origin.

METHODS AND MATERIALS

Test description



Seed Source Origin

- | | |
|--------------------------------|-------------------------------|
| 1 Coastal North Carolina | 4 Central Mississippi/Alabama |
| 2 Piedmont North Carolina | 5 Arkansas/Oklahoma |
| 3 Northern Mississippi/Alabama | |

Figure 1. Eight test locations of the Long-Term Seed Source Study are shown in relation to the northern boundary of the natural range of Loblolly pine (dashed line) and the seed source origin (shaded). A set of four tests were established in each of two years starting in 1982.

Eight test sites were planted with eight wind-pollinated families within each of five loblolly pine seed sources: coastal North Carolina (C-NC), piedmont North Carolina (P-NC), northern Mississippi/Alabama (N-M/A), central Mississippi/Alabama (C-M/A) and Arkansas/Oklahoma (A/O). The families in each source were the best available for volume production from first generation seed orchards. The trees were protected from insect attack for the first three years. Also, competition from hardwoods was kept to a minimum.

Measurements

Sweep, survival, fusiform rust infection, height, and diameter at 4.5 feet (1.37 meters) were measured at age eight years. Sweep was measured using a six-point ocular scoring method with each point being a one centimeter deflection from a eight-foot straight edge. The maximum deviation in the first twelve feet (3.66 meters) of the main stem, excluding the butt swell and lean, was scored. Each tree was thoroughly examined to locate the maximum deviation and quantitative measurements were periodically scored to maintain accuracy. Individual tree volume was calculated for inside bark of the entire stem (Smalley and Bower 1968).

Statistical analyses

An analysis of variance was used to test significant differences among seed sources, families and G x E interactions. Each test was planted in a split-block design with the five seed sources as main plots and eight wind-pollinated families within each seed source as five-tree row plots.

$$Y_{ijklm} = \mu + L_i + B(L)_{ij} + S_k + F(S)_{l(k)} + S \times L_{ki} + S \times B(L)_{ijk} + F(S)_{l(k)} \times L_i + \varepsilon_{ijklm}$$

where:

Y_{ijklm}	value of tree m in family l , seed source k within block j block at location i
μ	experimental mean
L_i	effect of the i th location
$B(L)_{ij}$	effect of the j th block within i th location
S_k	effect of the k th seed source
$F(S)_{l(k)}$	effect of the l th family within the k th seed source
$S \times L_{ki}$	effect of the seed source by location interaction
$S \times B(L)_{ijk}$	effect of the seed source by block within location interaction
$F(S)_{l(k)} \times L_i$	effect of the family within seed source by location interaction
ε_{ijklm}	pooled error term

Variances associated with each effect were derived from the expected mean squares and the average genetic correlation among environments associated with families within seed sources was calculated as follows:

$$r_{f(s)} = \frac{\sigma^2_{f(s)}}{\sigma^2_{f(s)} + \sigma^2_{f(s)} \times l}$$

where $\sigma^2_{f(s)} + \sigma^2_{f(s) \times l}$ = family within seed source and family x location interaction variance components respectively.

The genetic correlation among sites was adjusted for interactions associated with heterogeneity (not due to true rank changes) of family variance components among sites:

$$r'_{f(s)} = \frac{\sigma^2_{f(s)}}{\sigma^2_{f(s)} + [\sigma^2_{f(s) \times l} - \text{var}(\sigma_{f(s)i})]}$$

where $\text{Var}(\sigma_{f(s)i})$ is the variance of the $\sigma^2_{f(s)}$ for locations $i = 1$ to 8

The average genetic correlation (and adjusted correlation) among environments associated with seed sources was calculated similarly:

$$r_s = \frac{\sigma^2_s}{\sigma^2_s + \sigma^2_{s \times l}} \quad r'_s = \frac{\sigma^2_s}{\sigma^2_s + \sigma^2_{s \times l} - \text{Var}(\sigma_{si})}$$

Because family x location interaction was statistically significant at the 1% level or higher for volume and sweep, the Finlay and Wilkinson (1963) method of regressing family mean against an environmental index was used to determine whether some families were more or less stable than others. To test for linearity, we used the R^2 from the slope values from the regression analysis. Any R^2 greater than 0.80 was considered high (McKeand, et al. 1988). With large numbers of families there was little value in recalculating the site mean after subtracting each family mean value in question as is recommended when only a few families are used.

$$X_{ij} = X_i + B_j (X_{.j} - X_{..}) + \epsilon_{ij}$$

where:

X_{ij}	mean of family i at environment j ($i=1$ to 40; $j=1$ to 8)
X_i	mean of family i over all environments
B_j	coefficient for family mean regressed against environment mean
$X_{.j}$	mean of all families over all environments
ϵ_{ij}	deviation or residual from a linear response

RESULTS AND DISCUSSION

Sweep exhibited more G x E interaction at the seed source level than at the family within seed sources level (Table 1). In contrast, volume appears to have slightly less G x E interaction with seed sources than families within seed sources (Table 1). A closer look at interactive seed sources and families are more relevant to decision-making in a seed source movement program than the analyses of variance alone.

Table 1. Genetic correlations among sites at the seed source (r_s) and family within seed source ($r_{f(s)}$) levels. The prime (') indicates that the correlations were adjusted for scale effects.

Trait	$r_{f(s)}$	$r'_{f(s)}$	r_s	r'_s
Sweep	0.86	0.89	0.10	0.15
Volume per Tree	0.68	0.74	0.80	0.85

Seed Sources

Seed sources for both sweep and volume at age eight years were generally stable across diverse physiographic areas with a few notable exceptions. For sweep, the coastal North Carolina source performed well for sweep across sites except at the two most crooked at Wahalak and Webster County, Mississippi. The decrease in rank was not related to longitude, latitude or proximity to the edge of the species range. Also, the central Mississippi/Alabama families showed a slight drop in rank with some sinuous stem growth observed when planted northwest of their origin. The piedmont North Carolina source was generally the straightest across all sites with central Mississippi/Alabama as the most crooked (Table 2). No genetic entries were severely maladapted with respect to volume production.

Table 2. Seed source means for sweep (cm) at age eight years across eight locations. Ranks in parenthesis range from the straightest (1) to the most crooked (5). Locations are ordered from least to most sweep.

Seed Source	Battiest, OK.	Smithville, OK.	Dequeen, AR.	Bruce, MS.	Scooba, MS.	Paron, AR.	Webster Co., MS.	Wahalak, MS.
NC-P	1.96 (1)	2.37 (2)	2.56 (3)	3.04 (4)	2.77 (1)	3.06 (1)	3.15 (1)	3.71 (1)
NC-C	2.26 (2)	2.06 (1)	2.15 (1)	2.55 (1)	2.83 (2)	3.29 (3)	3.59 (5)	4.16 (5)
MA-N	2.27 (3)	2.57 (4)	2.53 (2)	2.97 (2)	2.97 (3)	3.12 (2)	3.22 (2)	4.00 (2)
A/O	2.32 (4)	2.49 (3)	2.85 (4)	3.23 (5)	3.23 (5)	3.60 (5)	3.38 (3)	4.04 (3)
M/A-C	2.36 (5)	2.72 (5)	2.86 (5)	3.04 (3)	3.19 (4)	3.35 (4)	3.51 (4)	4.12 (4)

There were relatively small seed source differences in sweep means at this age but they were statistically important and unrelated to volume or survival (Table 3) at age eight years. Seed sources which exhibited high volume such as central Mississippi/Alabama had as much sweep as a seed source with considerably less volume such as Arkansas-Oklahoma. Coastal North Carolina was first for volume production and second for sweep. Although the Arkansas/Oklahoma and central Mississippi/Alabama both displayed more sweep than other seed sources, Arkansas/Oklahoma had the best survival across all sites with central Mississippi/Alabama having the worst.

Table 3. Seed source means across eight locations for individual-tree volume (dm^3), sweep (cm), and cumulative percent mortality at age eight years. Standard errors of the means are in parentheses.

Source	Mean Volume - dm^3 (STDERR)	Mean Sweep - cm (STDERR)	Cumulative Mortality
Coastal North Carolina	$28.24 \pm (.30)$	$2.83 \pm (.03)$	6.0%
Central Mississippi/Alabama	$26.93 \pm (.29)$	$3.13 \pm (.03)$	8.1%
North Mississippi/Alabama	$25.75 \pm (.29)$	$2.93 \pm (.03)$	5.7%
Piedmont North Carolina	$24.43 \pm (.27)$	$2.80 \pm (.03)$	5.1%
Arkansas/Oklahoma	$22.60 \pm (.25)$	$3.12 \pm (.03)$	3.7%

Seed source stability across sites was tested using the Finlay and Wilkinson (1963) method of regressing the environmental source mean against the location source means (all R^2 values were greater than 0.90). A regression slope of $b=1.0$ indicates an average stability across sites and a response in direct proportion to site change. A slope of $b > 1.0$ indicates a source is unstable and that it is more responsive to site changes. A slope of $b < 1.0$ indicates the source is stable and that it is less sensitive to site changes.

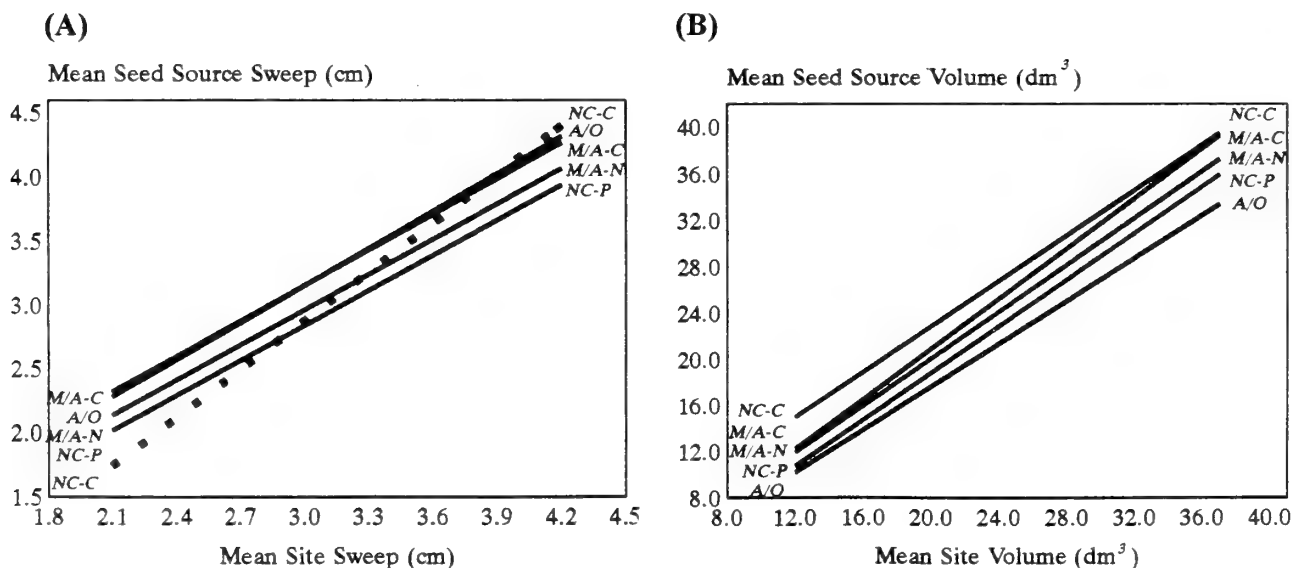


Figure 2. A) Seed source regression slope (b) for sweep at age eight years across eight locations, B) Seed source regression slope (b) for volume at age eight years across eight locations.

For sweep, coastal North Carolina proved to be the least stable across physiographic areas (Figure 2A). In contrast, the Arkansas/Oklahoma seed source was stable but exhibited poor sweep. The central Mississippi/Alabama seed source was generally stable at all locations except the northeastern Arkansas and Oklahoma sites where it dropped slightly in rank. Piedmont North Carolina and northern Mississippi/Alabama sources showed average stability for sweep and were generally the straightest across test locations (Figure 2A). All seed sources were stable across locations for volume (Figure 2B).

Families within seed sources

Stability of families within seed source was also tested using the Finlay and Wilkinson (1963) method of regressing the environmental family means against the location family means. Of the 40 families, 32 had R^2 values higher than 0.80. Of the remaining eight families with R^2 values which fell between 0.54 and 0.80, four of these were the extremely straight piedmont North Carolina families which exhibited negligible variation for sweep. In addition, 17 out of 40 families had slope (b) values for sweep which were significantly different than $b=1$ at the 10% level. Of these 17 families, 10 came from the more unstable stable seed sources: coastal North Carolina and central Mississippi/Alabama.

For volume, families with stabilities that are responsive to site conditions are desired to maximize productivity through intensive silvicultural practices. In contrast, when it is uncertain what factors directly influence sweep, selecting a family exhibiting straight stems and average stability ($b=1$) may be more advisable.

There were several interesting exceptions for sweep at the family level. One top-ranking coastal North Carolina family for volume proved to be highly unstable for sweep and quite crooked (Figure 3A). However, four coastal North Carolina families were well-adapted to non-local sites with high volume production and exceptionally straight stems.

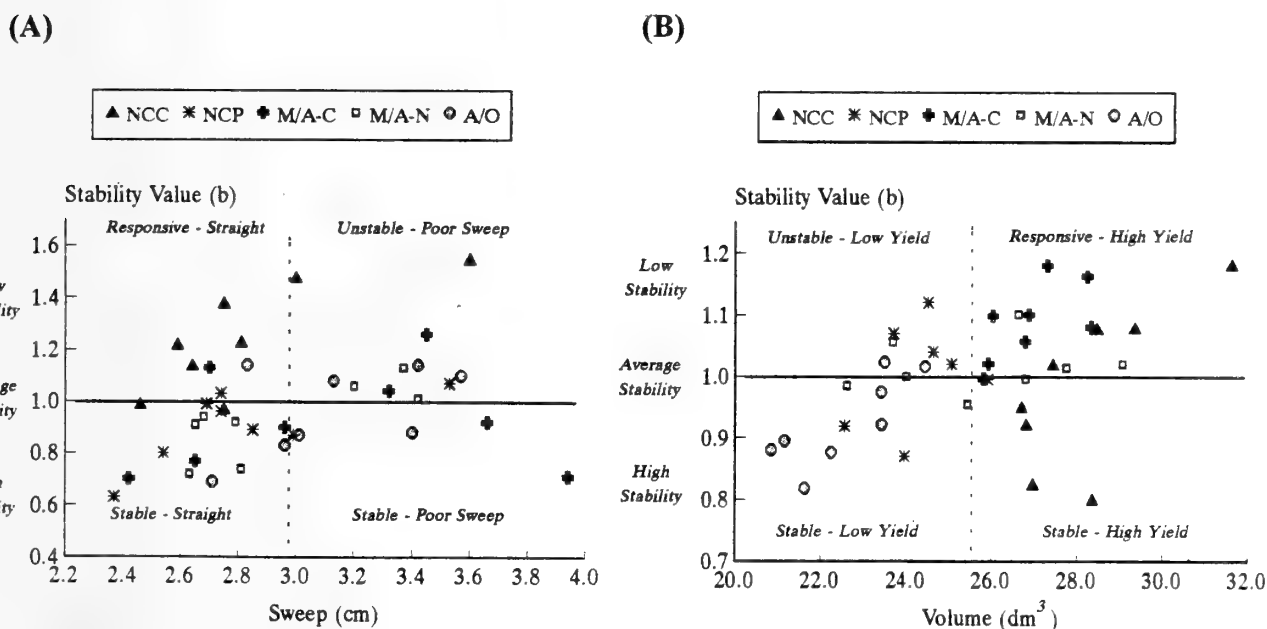


Figure 3. A) Family stability for sweep at age eight years across eight locations, B) Family stability for volume at age eight year across eight locations. Each point on the graph represents an open-pollinated family within the indicated seed sources.

Central Mississippi/Alabama demonstrated the widest spread in sweep among families (range=1.5 cm) (Figure 3A). Some families within this seed source were among the straightest and most stable in the tests while others were very unstable and crooked (Figure 3A). Three other sets of families, northern Mississippi/Alabama, piedmont North Carolina and Arkansas/Oklahoma were generally stable with family performance in sweep overlapping among seed sources (Figure 3A).

Results differed greatly at the family level between volume and sweep. For volume, central Mississippi/Alabama families responded well to site conditions favoring high volume production (Figure 3B). In contrast, sweep had more variability among families. The coastal North Carolina families generally were stable to responsive and were among the best for both growth and straightness. Piedmont North Carolina, northern Mississippi/Alabama, and Arkansas/Oklahoma families also exhibited high to average stability with less respect to volume production.

There was no correlation among families for sweep and volume (Figure 4). Therefore, it is possible to select for both high volume production and straight stems among families within seed sources.

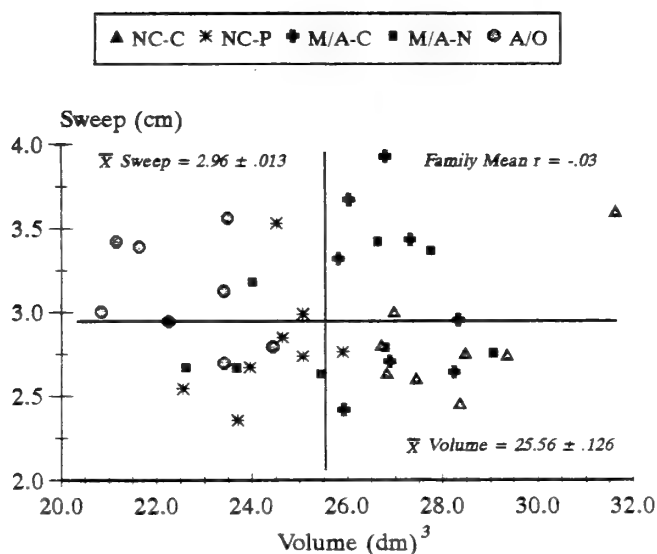


Figure 4. Scatter diagram of family mean for sweep and individual tree volume at age eight years across eight locations.

CONCLUSIONS

- For sweep, there was more G x E at the seed source level than for families within seed sources.
- For volume, it was reversed with slightly less G x E for volume at the seed source level than for families within seed sources.
- None of the seed sources or families exhibited serious maladaptation on any of the eight sites for sweep at age eight years.

- There was no correlation between sweep and volume at the seed source or family level.

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COMPUTER MODELING OF A SUBLINING BREEDING SYSTEM

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Abstract.-- Many tree breeding programs have adopted some form of multiple population strategy (sublining) to manage inbreeding. Many questions arise about how these populations should be constructed and managed. Among these are the questions of how individuals should be assigned to sublines and how selection should be done within and among sublines. Computer simulation of a sublining breeding strategy suggests that: (1) selecting the best individual from the four best families (self or outcross) ranked on expected breeding values is an alternative that will give good genetic gains and result in relatively moderate rates of increase in coancestry within sublines; (2) assigning parents to sublines at random or disassortatively rather than by positive assortment will increase within-subline genetic variance and result in greater expected genetic gains.

Keywords: Positive assortative mating, disassortative mating, random mating, elite populations.

INTRODUCTION

The North Carolina State University-Industry Cooperative Tree Improvement Program adopted a breeding strategy in 1992 for the third cycle of selection and breeding. The strategy aims to provide maximum genetic gains in the short term as well as to maintain genetic diversity to ensure the viability of breeding populations in the long term. The details of the strategy may be found elsewhere (McKeand and Bridgwater 1992), but the fundamental population structure is a hierarchy of three populations. A **mainline breeding population** will have about 160 parents available for each Cooperative member and will be maintained in small sublines (size 4, in the plan) primarily to provide for long term genetic gains. The most intensively selected and managed level in the hierarchy will be **elite populations** of about 40 parents. The elite populations will be bred as rapidly as possible to provide maximum short-term genetic gains. A third level in the population hierarchy will be extreme genotypes maintained as a **genetic diversity archive**.

The goal is to cycle elite populations as rapidly as possible. Since production populations will be derived from these, genetic gains will be realized from plantations sooner than if larger populations, requiring more time and effort, were used. Elite populations may be managed in a variety of ways and will be structured to meet the needs of individual or groups of cooperative members. One option under evaluation is to subdivide elite populations into sublines as small as 4 parents each and to mate these in diallels which will include self-fertilization. This action will result in inbreeding and an increase in homozygosity at a rate that depends on the method of selection employed. When inbreeding reaches levels that require reduction, the plan is to enrich the elite populations from the much larger mainline population which will have been bred at a slower rate. The greater selection intensity possible in the larger mainline populations is expected to provide material suitable for inclusion in the elite populations. Introductions from the mainline populations will reduce coancestry in elite populations.

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Regular systems of breeding in small populations have been examined in great detail elsewhere (Falconer 1989). However, in reality, breeding populations are managed in a much more eclectic manner than theoretical breeding schemes. We have used computer modeling to simulate alternative selection and mating schemes that breeders might use to manage multiple populations in sublines.

MODEL CHARACTERISTICS

Genetic Model

The genetic model permits variable numbers of loci up to 128 in multiples of 8 loci. At present, all loci act independently and have only two alternative alleles (0 or 1) at a locus. The genetic value assigned to an allele is a variable specified when the population is generated. At present, all alleles of the same type have the same value.

Mainline Population Generation

Populations of up to 500 individuals can be generated (Figure 1). Each individual in the population is generated by randomly assigning an allele to each locus. Gene frequencies may be varied when the population is generated, but were made 0.5 in the base population (Generation 0) for this simulation. A genotypic value was calculated for each individual by summing gene values over loci. Total genetic variance (V_g) was assumed to be the sum of additive (V_a) and dominance (V_d) variances, that is, $V_g = V_a + V_d$. Variances due to epistasis, linkage, and linkage disequilibrium were assumed to be zero. Since populations were at equilibrium only at generation 0, genetic variances at each locus were calculated from genotype frequencies, rather than gene frequencies:

Genotype	A_1/A_1	A_1/A_2	A_2/A_2
Value	G_{11}	G_{12}	G_{22}
Frequency	P_{11}	P_{12}	P_{22}

Fitting the model $G_{ij} = u + a_1 + a_2$ (where u = the overall mean, a_1 and a_2 are the average effects of A_1 and A_2) by least squares minimizes:

$$Q = P_{11}[G_{11}-u-2a_1]^2 + P_{12}[G_{12}-u-a_1-a_2]^2 + P_{22}[G_{22}-u-2a_2]^2$$

and the genetic variances are:

$$V_a = P_{11}(2a_1)^2 + P_{12}(a_1+a_2)^2 + P_{22}(2a_2)^2 \text{ and,}$$

$$V_d = P_{11}(G_{11}-u-2a_1)^2 + P_{12}(G_{12}-u-a_1-a_2)^2 + P_{22}(G_{22}-u-2a_2)^2.$$

The expressions for the a_i are:

$$a_1 = -a_2(P_{12}+P_{22})/(2P_{11}+P_{12}) \text{ and } a_2 = -a_1(2P_{11}+P_{12})/(P_{12}+2P_{22}).$$

Phenotypic values may be assigned to individuals in one of two ways. A narrow-sense heritability (h^2) can be specified and the environmental variance (V_e) is calculated from:

$$V_p = V_a/h^2 \text{ and } V_e = V_p - V_g$$

or V_e can be specified, in which case:

$$V_p = V_e + V_g \text{ and } h^2 = V_a/V_p.$$

Individuals generated in the mainline population may be ranked by breeding value, genotype, phenotype, or at random and are saved to a file that is used as input to the breeding strategy model.

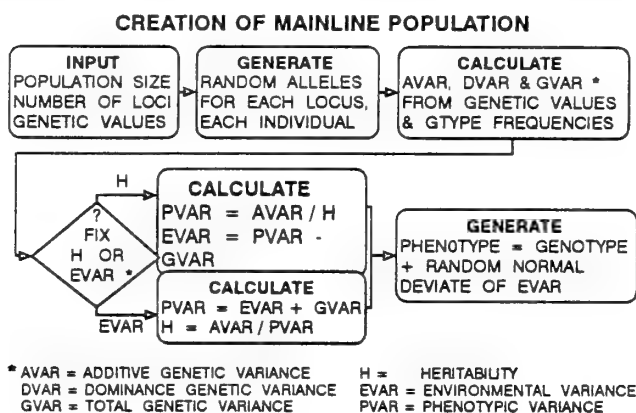


Figure 1. Flow Diagram for the generation of a mainline population.

Breeding Strategy Model

Elite populations may be generated in different ways by selecting a subset of the mainline population. That is, if the top quartile of the mainline population based on breeding values were to be included in an elite population, it would be necessary to generate a mainline population ranked by breeding values. Then the top quartile should be selected for the breeding strategy model.

After a mainline population is generated, the breeding strategy model (Figure 2) permits the assignment of individuals to sublines in different ways. Three possibilities are: (1) Positive assortative mating (PAM) (1,2,3,4), (5,6,7,8), (n-3,n-2,n-1,n); (2) Dissassortative mating (DAM) (1,2,n-1,n), (3,4,n-3,n-2), (n/2-2,n/2-1,n/2+1,n/2+2); and (3) Random mating (RAM) (Assigned to 4-parent diallels at random), where 1,2,3,4, n imply ranks based on expected mid-parent values.

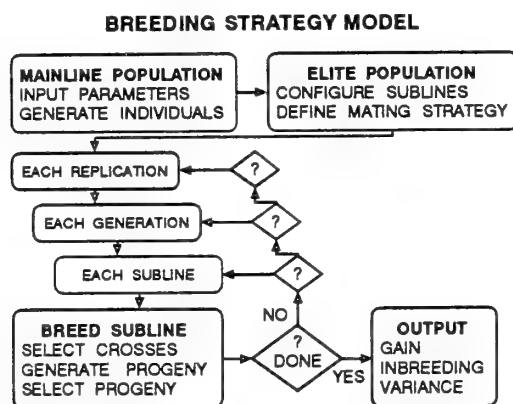


Figure 2. Flow Diagram for the breeding strategy model.

Selection within sublines is done in each generation by ranking each parent and crosses among them based on expectations from their breeding values (Figure 3). Individuals within progeny groups are generated by random segregation at each parental locus and are selected based on phenotypes. An allele identifier is assigned to each allele at each locus to facilitate the calculation of inbreeding coefficients ("F").

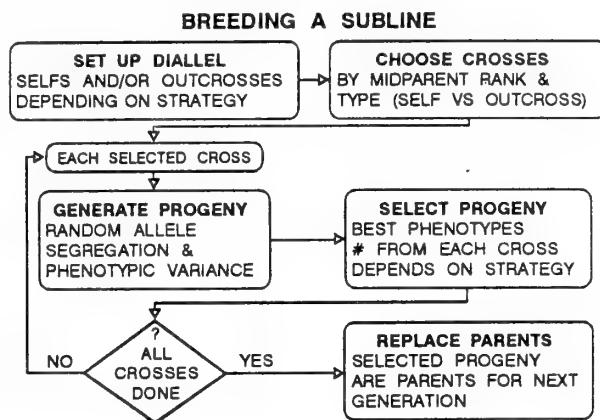


Figure 3. Mating a Subline

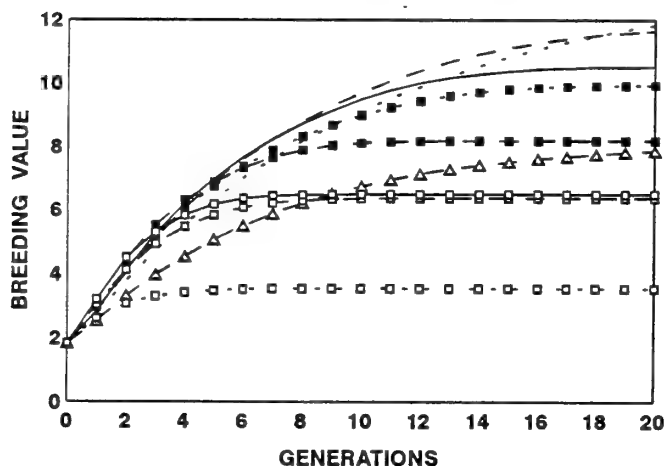
Different schemes for mating the selected individuals may be designated. To date we have evaluated nine selection and mating schemes that represent a broad array (Figure 4). Reports may be generated for each replication, generation, or subline, or all three. Statistics generated by the model within and among sublines and for the population total include: (1) Additive, dominance, and total genetic variances, (2) Mean genotypic value, (3) Mean breeding value (Calculated by mating each individual to a standard check population with equal gene frequencies), (4) Genetic gain in breeding value (BV in generation n - BV in generation $n-1$), (5) Inbreeding coefficient ("F") (Correlation among alleles "identical by descent"), and (6) Variances for numbers 1-5 above over replicate runs.

RESULTS AND DISCUSSION

Comparison of Selection Schemes

The 9 selection schemes in Table 1 were compared based on their mean breeding values (Figure 4) and mean inbreeding coefficients (Figure 5) for 20 generations. A mainline population of 160 entries was generated using an $h^2 = 0.2$ and a degree of dominance = 0.5. Parents with the 40 best breeding values were included in the elite population. For the purposes of this comparison, parents were assigned at random to 4-parent sublines in generation 0. The 4 selected parents in each generation were mated in 4-parent disconnected diallels with self-matings and 100 progeny were generated per mating.

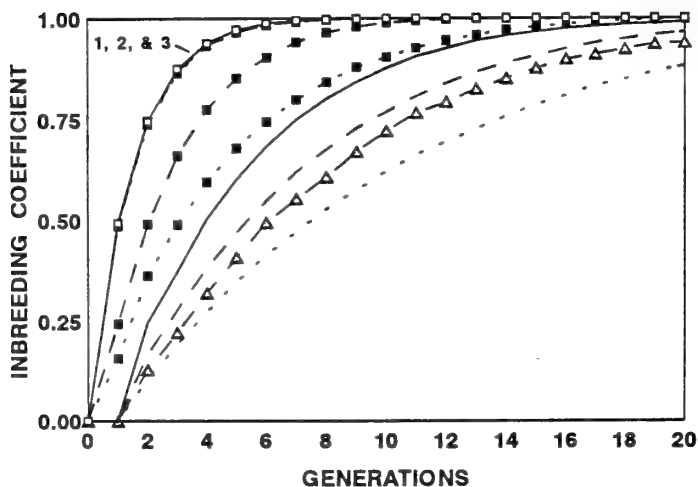
Mating schemes 1, 2, and 3 assigned 4, 2, or 1 S1 progeny from 1, 2, or 4 parents, respectively, to a 4-parent subline. Breeding values plateaued after 6 to 8 generations (Figure 4) and inbreeding coefficients increased very rapidly to over 0.9 in 4 generations (Figure 5). The most extreme of these (Method 3) is effectively maintaining 40 selfed lines through time. Mean breeding values were calculated by mating each individual in each subline to an equilibrium population and averaging the individual breeding values for each subline and over sublines. Thus, mean breeding values illustrate the potential for gain in a production population formed in such a way that individuals from different sublines were not allowed to produce progenies. That is, mean breeding values do not reflect the reduced vigor and seed yields that will arise with increased coancestry in the breeding populations. It may, therefore, be wise not to use such extreme methods of selection and mating.



MATING SCHEMES

- 1 —□— 1 SELF Family, 4 Ind.
- 2 —□— 2 SELF Families, 2 Ind.
- 3 - -□- - 4 SELF Families, 1 Ind.
- 4 ——— 1 OUTCROSS Family, 4 Ind.
- 5 - - - 2 OUTCROSS Families, 2 Ind.
- 6 -△- 2 UNRELATED Families, 2 Ind.
- 7 -■- 2 ANY TYPE Families, 2 Ind.
- 8 - - - - 4 OUTCROSS Families, 1 Ind.
- 9 - -■- - 4 ANY TYPE Families, 1 Ind.

Figure 4. Mean breeding values for an elite population of 40 selected from a mainline of 160, selected by nine different methods.



MATING SCHEMES

- 1 —□— 1 SELF Family, 4 Ind.
- 2 —□— 2 SELF Families, 2 Ind.
- 3 - -□- - 4 SELF Families, 1 Ind.
- 4 ——— 1 OUTCROSS Family, 4 Ind.
- 5 - - - 2 OUTCROSS Families, 2 Ind.
- 6 -△- 2 UNRELATED Families, 2 Ind.
- 7 -■- 2 ANY TYPE Families, 2 Ind.
- 8 - - - - 4 OUTCROSS Families, 1 Ind.
- 9 - -■- - 4 ANY TYPE Families, 1 Ind.

Figure 5. Mean inbreeding coefficients ("F") for an elite population of 40 selected from a mainline of 160, selected by nine different methods.

Methods 4, 5, and 8 represent 1, 2, and 4 of 6 full-sib families saved, excluding only the selfs. The full-sibs may also be related as half-sibs. Method 6 specifies unrelated families, implying that 2 individuals were chosen from each of crosses 1x2 and 3x4, where the parent number is the rank based on breeding values. Methods 7 and 9 are the simplest in that they permit selection of the 2 or 1 best individuals from each of the 2 or 4 best families of any type. Relationships are disregarded and selection from selfed lines is permitted.

Methods 7 and 9 have higher mean breeding values than 4, 5, and 8 through at least 3 generations, but plateau and reach higher values of "F" sooner. Since the Cooperative's plan is to enrich elite populations from the mainline populations, method 7 or 9 may be the best for selecting and mating in elite populations. The more conservative choice of these two methods is 9 which reached high values of "F" later (Figure 5). However, both the rate of increase in mean breeding value and "F" depend upon being able to produce selfed progenies for each entry. Since it is known that all parents do not produce selfed progenies with equal ease, we examined the more realistic assumption that only 50% of the parents in each generation would produce an adequate number of selfed progenies to permit selection among them. We assumed no correlation between breeding values and the ability to produce selfed progenies, and simply assigned a probability of 0.5 that a selfed progeny could be selected from a parent regardless of its breeding value. The differences in mean breeding values were insignificant through 10 generations. Furthermore, "F" increased more slowly when selfed progenies could be selected for only half the parents.

Comparison of Methods for Subline Assignment

We next examined the impact of method of assigning parents to sublines in generation 0. For the purposes of this examination, we used the same mainline populations of 160 and 40 generated as above. The methods of assignment to sublines were those described in the section on "Breeding Strategy Model", above. We used only mating method 9 with 50% selfing success in this analysis.

Production populations will be formed from the elite population in a way that prevents individuals from different sublines from producing progeny. Thus, selection will be largely, if not exclusively, within rather than among sublines. Given that, it should be desirable to favor assignment schemes that promote increased additive genetic variance within sublines. A comparison of mean breeding values for PAM, DAM, and RAM shows that mean breeding values increase more from generation 0 to 1 for DAM and RAM than for PAM (Figure 6). This difference arose because the within-subline additive genetic variances was greater for DAM and RAM in generation 0. This difference occurred in both population sizes, but was much smaller for the population size 40, which is a selected subset of the larger population of 160. Thus, positive assortment of parents to sublines can be expected to give smaller genetic gains than either dissasortative or random assignment.

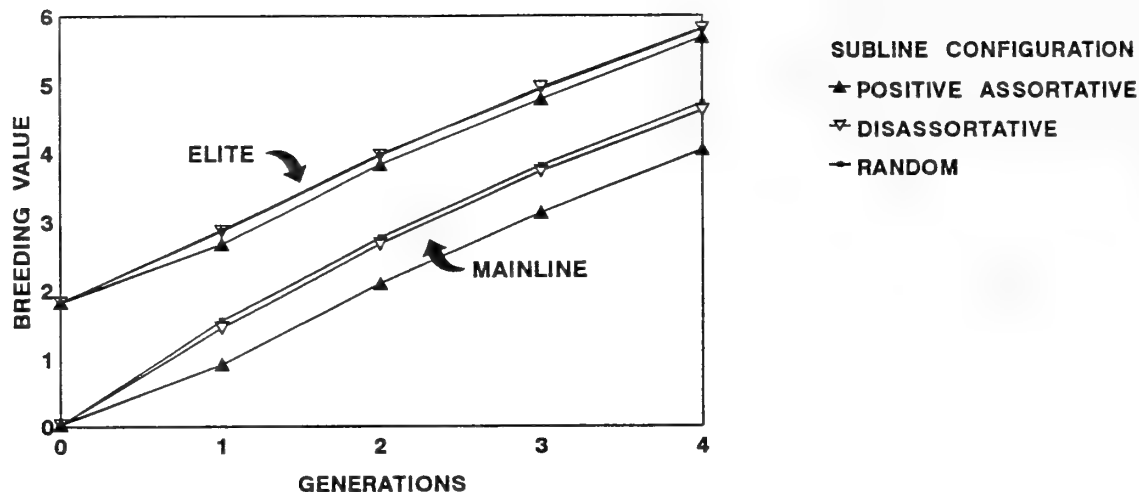


Figure 6. Mean breeding values for a mainline (160) and an elite (40) population

SUMMARY AND CONCLUSIONS

Computer modeling different aspects of a sublining breeding strategy suggests that: (1) selecting the best individual from the four best families (self or outcross) ranked on expected breeding values is an alternative that will give good genetic gains and result in relatively moderate rates of increase in coancestry within sublines; (2) assigning parents to sublines at random or disassortatively rather than by positive assortment will increase within-subline genetic variance and result in greater increases in breeding value for the first generation of selection and mating.

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MODELING LOBLOLLY PINE AGE-AGE CORRELATION FOR HEIGHT
USING THE DEGREE OF NON-DETERMINATION.F. H. Kung¹

Abstract.--Growth is a collective process. If we can assume that the error component of predicting growth from year to year is homogenous and additive, then the degree of non-determination ($1-r^2$) between height in two years is a linear function of the age difference. We used data from the South-wide Loblolly Pine Provenance Test to calculate the age-age correlations for height growth from age 3, 5, 10, 15, 20 and 25 years. The degree of non-determination (DON) then was computed from each correlation. The DON model and its derived non-linear model were then compared with two other models: Lambeth model and response surface model. The DON model had the greatest F value (416.56) and the largest r-square (0.956). After transformation from DON to correlation, the predictive correlation from the DON model proved to be closer to the observed correlation than other models. The DON model suggests that about 2.5% of accountability in height growth among loblolly pine provenances is lost in each subsequent year.

Keywords: *Pinus taeda* L., juvenile-mature correlation, selection.

INTRODUCTION

Tree improvement programs involve long-term investment. We would like to make selections as early as possible to reduce the breeding cycle and to maximize efficiency of land use. For example, knowing that oleoresin yields of various slash pine progeny correspond closely with the yields of their parents, enables us to use short-term progeny test of 3-year rotation and 3 feet spacing for parental selection (Squillace and Gansel 1968). The efficacy of early selection is related to the correlation between early and late assessment of the trait being improved (Kung 1975). Early selection is usually less effective, but may be more efficient than late selection in terms of genetic gain per unit of area and per unit of time (Kung 1973, Bohren 1975).

Given N repeated measurements at various ages, N*N correlations are possible in the age-age correlation matrix. For example, the original South-wide Loblolly Pine Seed Source Study (Wells and Wakeley, 1966, Nance and Wells, 1981) was measured at age 3, 5, 10, 15, 20, and 25 years (Table 1), and therefore, there are 36 correlations in the matrix (Table 2). Because the matrix is symmetrical and the values on the diagonal are unity, usually only a triangular matrix is reported in literature. The age-age correlation becomes smaller as the difference between two ages becomes greater. Taking a logarithm transformation of age, Lambeth (1980) found a linear relationship between the difference of two transformed ages and its age-age correlation. The Lambeth model is expressed as follows:

$$r = a + b \text{ (LAR)} \quad . \quad . \quad . \quad . \quad (1)$$

Where

r = correlation coefficient

a, b = regression coefficient

LAR = logarithm of age ratio = $\log(\text{young age}/\text{old age})$

$$= \log(\text{young age}) - \log(\text{old age})$$

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Table 1. Mean height of loblolly pine provenances at various ages.

No.	Provenance	Mean height at age (years)					
		3	5	10	15	20	25
-cm-							
C-301	E. Maryland	137	331	875	1272	1606	1856
C-303	SE. N. Carolina	138	332	886	1305	1642	1932
C-305	E. N. Carolina	145	346	920	1353	1701	1953
C-307	W. S. Carolina	119	288	782	1190	1547	1800
C-309	SE. Georgia	146	341	873	1269	1624	1921
C-311	NE. Georgia	118	286	792	1205	1568	1817
C-315	N. Alabama	131	317	827	1245	1564	1830
C-317	NE. Alabama	116	282	767	1164	1502	1787
C-319	N. Alabama	139	322	850	1253	1598	1849
C-321	NE. Mississippi	114	275	775	1188	1516	1837
C-323	SE. Mississippi	131	328	883	1293	1620	1878
C-325	E. Texas	133	319	851	1225	1525	1782
C-327	SW. Arkansas	125	302	808	1152	1476	1731
C-329	W. Tennessee	119	284	794	1151	1472	1719
C-331	NW. Georgia	105	275	787	1193	1497	1813

Table 2.--Correlation matrix and degree of non-determination matrix for tree heights at age 3, 5, 10, 15, 20 and 25 years.

Correlation Matrix						
Age in Years						
Age	3	5	10	15	20	25
3	1.00000	0.96739	0.90097	0.79722	0.79064	0.65974
5	0.96739	1.00000	0.96333	0.87443	0.83263	0.71456
10	0.90097	0.96333	1.00000	0.92528	0.85869	0.75366
15	0.79722	0.87443	0.92528	1.00000	0.96427	0.91275
20	0.79064	0.83263	0.85869	0.96427	1.00000	0.93072
25	0.65974	0.71456	0.75366	0.91275	0.93072	1.00000
Degree of Non-determination Matrix						
Age in Years						
Age	3	5	10	15	20	25
3	0.00	0.06	0.19	0.36	0.37	0.56
5	0.06	0.00	0.07	0.24	0.31	0.49
10	0.19	0.07	0.00	0.14	0.26	0.43
15	0.36	0.24	0.14	0.00	0.07	0.17
20	0.37	0.31	0.26	0.07	0.00	0.13
25	0.56	0.49	0.43	0.17	0.13	0.00

When we examine a square correlation matrix (Table 2), we can also see that the correlation becomes smaller as the distance from a cell to the diagonal becomes greater. Therefore, the correlation matrix may be viewed as a symmetrical response-surface model with a ridge on the diagonal and with slopes incline toward two corners. A general response-surface model in analytical geometry is usually expressed as

$$Z = aX^2 + bXY + cY^2 + dX + eY + f$$

Because of symmetry, it is necessary to have coefficients $a=c$ and $d=e$. Furthermore, contrary to mathematicians, statisticians like to order variables by ascending power. Thus, a response-surface regression model for age-age correlation may be expressed as follows:

$$r = a + b (X+Y) + c (X^2 + Y^2) + d (X*Y)$$

Where r = correlation between age X and age Y
 a = intercept
 b = coefficient for linear terms
 c = coefficient for quadratic terms
 d = coefficient for crossproduct term.

In a previous study, we have successfully fitted a symmetrical response-surface model to stem volume data obtained from stem-analysis of 51 cryptomeria trees between age 3 to 30 years (Yang and Kung 1987). Since we have found that coefficient b was not significant, and that coefficient d was twice the size of coefficient c but had a negative sign, the above 4-coefficient model can be shorten to a 2-coefficient model with little reduction in the coefficient of determination:

$$r = a + b (X - Y)^2$$

If we define difference in ages (DA) as $X - Y$, then

$$r = a + b (DA)^2 \quad (2)$$

We will call this model by the name of DA2 model in this paper, representing linear relationship with the second power of age differences (DA).

Both the Lambeth model (1) and DA2 model (2) show the relationship between the correlation and the age difference but not why. A systematic model therefore is proposed to clarify this relationship.

THE DON MODEL

Growth is a collective process. The total height of a tree is completely determined by the previous height and the last increment, but the system controlling increment may have two types of elements: order and chaos. The orderly elements are the previous height and all earlier increments. The chaos element is a random and independent distribution of residual errors within a given specific growing interval.

The square of a correlation coefficient is called the R-square. The R-square in a regression model is called the degree of determination, and the quantity of $1-(R\text{-square})$ is call the degree of non-determination. The former indicates how much variance in terms of sum of squares (SSQ) can be explained by the regression model, and the latter, cannot be explained. The degree of non-determination (DON) therefore is the ratio of error SSQ divided by the total SSQ.

Because we cannot change history, we may assume that previous deviation from orderly growth is interminable. For example, if we compare a tree with accidental damage to the terminal bud with other non-damaged trees, the damaged

tree will deviate from the orderly pattern of height growth. The damage may be repaired but can never be denied. The deviation is recorded and cannot be erased.

Total growth is the accumulation of all previous annual growth and total error is the sum of all previous errors. Can we then assume that the degree of non-determination is also additive? In Table 2 we find in several cases that the additive property seems to be valid. For example, the DON from age 3 to age 5 is 0.06, from age 5 to age 25 is 0.49, the sum of these two is 0.55 which is close to the value 0.56 given by the DON between age 3 and age 25. In the second example, the fitting errors from age 3 to age 20 (DON=0.37) is the sum of those from age 3 to age 5 (DON=0.06), and from age 5 to age 20 (DON=0.31). In the third example, we have 0.07 (DON for age 5 to 10) + 0.43 (DON for age 10 to 25) = 0.50, the sum is again very close to the value of 0.49 (DON for age 5 to 25).

Further examination of the DON matrix in Table 2 suggests that DON has a linear relationship with age differences (DA). The average DON for a 5-year time lag is .13, for a 10-year lag is .22, and for a 15-year lag is .37. Therefore, the DON model can be expressed as:

$$\text{DON} = a + b (\text{DA}) \quad (3)$$

Because the DON model minimizes the error variance of DON, not the original correlation, would the following nonlinear model, transformed from the DON model fit the original correlation better?

$$r = \text{sqrt} [a + b (\text{DA})] \quad (4)$$

We will call this nonlinear model as NLIN in this paper.

STATISTICAL COMPARISON OF THE FOUR MODELS

The above four models were formulated by PROC REG and PROC NLIN (SAS Institute, Inc. 1987). The regression coefficients and their standard errors (listed below the coefficients) of the four models are as follows:

DON Model	(1-r ²) =	-0.004164 + 0.02473 * DA	
		[0.01246] [0.001211]	
or,	r =	(1.004164 - 0.02473 * DA) ^{0.5}	
DA2 Model	r =	0.971385 - 0.000699 * DA ²	
		[0.009257] [0.00005325]	
LAMBETH Model	r =	0.996399 - 0.13703 * LAR	
		[0.01440] [0.01459]	
NLIN Model	r =	(1.005177 - 0.024924 * DA) ^{0.5}	
		[0.014143] [0.001191]	

When there is no age difference (i. e. DA=0 or LAR=1), the true correlation is 1.000. The DON and the NLIN models overestimate this correlation by 0.0020 and 0.0025, the Lambeth and the DA2 model underestimate by 0.0036 and 0.0286 respectively. It is also interesting to know from the DON model that each year about 2.5% (0.02473) of unexplained variance is accumulated.

While F-test is not applicable to the nonlinear model, among the remaining three models, the DON model has the greatest F-value (Table 3). The Don model also has the greatest R-square when compared with other models. On the other hand, the Lambeth model has the smallest F-value, the largest MSQ error and SSQ error, and the largest predicted residual sum of squares.

The smallest error mean square and error sum of squares are found in the NLIN model. The DON model should not be compared here because it is not based on the original correlation but on the degree of non-determination.

Table 3.--Comparison of statistical analysis of four models.

Statistics	Regression Model			
	DON	DA2	LAMBETH	NLIN
F-value	416.56	172.55	88.27	na
MSQ model	0.61214	0.19569	0.17876	8.56043
MSQ error	0.00147	0.00113	0.00203	0.00052
R-square	0.9564	0.9008	0.8229	0.9548*
SSQ model	0.61214	0.19569	0.17876	17.12086
SSQ error	0.02792	0.02155	0.03848	0.00983
SSQ uncorrected total				17.13068
.SSQ corrected total	0.64007	0.21724	0.21724	0.21724
Predicted Resid SS	0.0331	0.0263	0.0467	

na: not applicable
 *: calculated from $1 - (\text{SSQ error} / \text{SSQ corrected total})$

PERFORMANCE COMPARISON OF THE FOUR MODELS

In order to have a valid evaluation of the four models, we should compare how good are the predictions. By inserting the ages into the four regression models and solving for the correlation, the predicted age-age correlations are listed in Table 4. If we rank the predictions from 1 to 4, 1 being the nearest to the observation, and 4, the remotest from the observation, then the DON model with a mean score of 1.667 is the best model (Table 5). The NLIN model is better than the Lambeth model, and the DA2 model is between the NLIN and the Lambeth models.

Using the absolute deviation between predicted and observed values as input variables in a two-way analysis of variance, we find no differences between DON and NLIN models, no differences between DA2 and Lambeth models, but differences between these two groups are highly significant. The mean absolute deviations are 0.016 for the DON and NLIN models, 0.028 for the DA2 model and 0.032 for the Lambeth model respectively.

CONCLUSIONS

Based on the South-wide Loblolly Pine Provenance Tests data, age-age correlations can be modelled successfully using the degree of non-determination (DON). The DON model is superior to the acclaimed Lambeth model because it has smaller fitting errors. The DON model indicates that the error component is accumulated at a rate about 2.5 percent each year.

Table 4.--Comparison of observed and predicted correlations in the four models.

Case	Age		Correlation				
	Age2	Age1	Obser.	Predicted by Model			
				DON	DA2	LAMBETH	NLIN
1	3	3	1.00000	1.0020*	0.9714	0.9964	1.0025
2	5	3	0.96739	0.9770	0.9686*	0.9264	0.9774
3	10	3	0.90097	0.9116	0.9371	0.8314	0.9114*
4	15	3	0.79722	0.8410	0.8707	0.7758*	0.8402
5	20	3	0.79064	0.7640	0.7692*	0.7364	0.7625
6	25	3	0.65974	0.6783	0.6328	0.7058	0.6759*
7	5	5	1.00000	1.0020*	0.9714	0.9964	1.0025
8	10	5	0.96333	0.9383	0.9539*	0.9014	0.9383
9	15	5	0.87443	0.8699*	0.9014	0.8458	0.8694
10	20	5	0.83263	0.7957	0.8140*	0.8064	0.7945
11	25	5	0.71456	0.7138*	0.6916	0.7758	0.7118
12	10	10	1.00000	1.0020*	0.9714	0.9964	1.0025
13	15	10	0.92528	0.9383*	0.9539	0.9408	0.9383
14	20	10	0.85869	0.8699	0.9014	0.9014	0.8694*
15	25	10	0.75366	0.7957	0.8140	0.8708	0.7945*
16	15	15	1.00000	1.0020*	0.9714	0.9964	1.0025
17	20	15	0.96427	0.9383	0.9539	0.9569*	0.9383
18	25	15	0.91275	0.8699	0.9014*	0.9264	0.8694
19	20	20	1.00000	1.0020*	0.9714	0.9964	1.0025
20	25	20	0.93072	0.9383*	0.9539	0.9658	0.9383
21	25	25	1.00000	1.0020*	0.9714	0.9964	1.0025

* Best predictor in each row.

Table 5.--Grouping of the four models by Duncan's multiple-range test.

Duncan Grouping		Mean Rank	Model
1] Using rank, ranking order 1 = prediction is the closest to observation, 4 = prediction is the remotest from observation.			
A		3.190	LAMBETH
A			
B	A	2.857	DA2
B			
B		2.286	NLIN
C		1.667	DON
2] Using absolute deviation between prediction and observation.			
A		0.03159	LAMBETH
A			
A		0.02787	DA2
B		0.01596	NLIN
B			
B		0.01576	DON

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COMPARISON OF CONTAINERIZED PROGENY TESTS
PLANTED IN SPRING AND FALL

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Abstract.-- Field observations have suggested that spring-planted progeny tests may be slower to develop than fall tests. If this delayed development is not soon outgrown, two potential problems of spring-planted tests must be considered; 1) growth and yield estimates may be biased downward from expectations of fall tests, and 2) family rankings may not be consistent across the two planting times. In this study, both loblolly and slash tests were evaluated to determine if either of the two potential problems appeared. Results suggest that after five years, effects of spring planting have been generally overcome in slash pine. However, there is a trend for spring-planted loblolly pine to show less growth. Performance in both species was also shown to be highly dependent on planting site. Whether or not significant changes occur in family rankings is more difficult to quantify. This study suggests that family x planting time interaction is not a serious concern.

Keywords: Pinus taeda L., P. elliottii Engelm., containerized seedlings, progeny testing, planting time.

INTRODUCTION

Many tree improvement programs are continuing to use containerized seedlings for the establishment of genetic tests. Barnett (1988) reviewed several of the advantages and disadvantages of containerized seedlings relative to bare-root stock, while van Buijtenen and Lowe (1981) summarized the existing and potential uses of containerized seedlings for establishment of progeny tests. In their presentation, van Buijtenen and Lowe (1981) suggested that only two time periods, early spring and early fall, were considered suitable for planting containerized tests. This restriction was based on such variables as time of expected freezes, soil moisture availability, and conflicting workload schedules.

Progeny tests established during the fall and spring time frames have generally been assumed to provide consistent data for both growth and yield information and family performance rankings. However, data to support these assumptions is lacking. An additional concern is that field observations of spring-planted seedlings often reveal a "champagne glass" appearance, where seedlings fail to break bud and grow as expected during the first summer after planting. Mexal and Carlson (1981) pointed out that this failure is a short term phenomena resulting from a incomplete satisfaction of a chilling requirement. After one winter in the field, the trees can then be expected to grow normally. However, this delayed development could lead to inaccurate conclusions regarding both total yield and family rankings.

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Previous studies involving time of planting have been limited to evaluation of survival and growth. For example, Barnett (1981) presented information for both slash pine (*Pinus elliotii* Engelm.) and loblolly pine (*P. taeda* L.) planted each month from January to September, 1973. His study, while designed to evaluate container types, generally showed better performance for spring-planted seedlings as opposed to fall-planted seedlings for both species. Data presented by Goodwin (1974) showed containerized seedlings of loblolly pine performed better if planted in spring rather than fall. However, in this study, several non-container related problems may have contributed to the poor showing of fall-planted trees. Goodwin et al. (1981) observed no difference in performance between containerized loblolly seedlings planted in April or September.

For a number of years, the Texas Forest Service (TFS) has utilized containerized seedlings of loblolly and slash pine in its testing program, with plantings being established in both spring and fall. This procedure allows for increasing the number of parents which can be tested in a given time frame, when considering a fixed greenhouse production capacity. However, the potential for spring-planted tests to develop slower than fall-planted tests warrants consideration. To further explore the use of containerized seedlings, several previously-established containerized tests were evaluated. The objectives of this evaluation were to; 1) determine if significant growth differences exist between tests established at the two different times (season of planting), and 2) to determine if family by date interaction is of sufficient magnitude to merit concern in the ranking of families for selection purposes.

METHODS

Seedlings for outplanting were produced by the Texas Forest Service at College Station, Texas utilizing the greenhouse production schedule indicated in Table 1. All seedlings were grown in commercially-available 163 cu.cm. containers using a 1:1 mixture of peat and vermiculite. Supplemental heating and lighting were provided the fall-sown seedlings (for spring planting). Tests were field planted as close to the indicated time as possible, although precise timing from year to year was not maintained. Once planted, tests were maintained according to standard procedures, to include periodic mowing, fireline construction etc.

Table 1. General production schedule for containerized seedlings planted in spring and fall.

<u>Activity</u>	<u>Fall-Planted</u>	<u>Spring-Planted</u>
Seed stratification (loblolly)	March	September
Sowing into containers	April	October
Greenhouse management	May-September	November-March
Outplanting	October-November	April-May

A total of 20 progeny tests established by the Texas Forest Service since 1980 were evaluated (Table 2). Of these tests, 8 were planted in the fall and 12 were planted in the spring.

Table 2. Number of containerized tests by location, species and time of planting.

Location	County	----Loblolly----		-----Slash-----	
		Fall	Spring	Fall	Spring
Magnolia Springs	Jasper	-	-	1	-
Hudson	Angelina	1	-	-	-
Fastrill	Cherokee	2	-	-	-
Pine Valley	San Jacinto	1	1	-	2
San Augustine	San Augustine	1	3	-	2
Siecke	Newton	-	-	1	3
Spurger	Tyler	-	-	1	1
Total		5	4	3	8

Plantings were measured for total height and diameter after five years in the field. Average family volume was also calculated using height and diameter and included dead and missing trees. Survival obtained at the end of the first year was analyzed using the arcsin \sqrt{x} transformation (Snedecor and Cochran, 1967). It should be emphasized that these tests were established with the objective of evaluating selected material and not the determination of optimum time of planting. Thus, confounding often occurs across families, planting location, year of planting and planting time (fall vs spring).

Three analysis were performed and were designed to; 1) indicate if growth differences could be observed for the two planting times, using as a data base all 20 plantings, 2) evaluate if planting time affects growth or if family x time interaction occurs, using plantings which had specific genetic checklots in common, and 3) to determine if a family x time interaction is present between two plantings with families in common. The first two of these analyses were performed for both loblolly and slash pine, with the third using only two slash pine tests.

RESULTS

Performance by planting date

The first analysis utilized all 20 progeny test plantings, with family means for survival (transformed) and five-year growth variables as input data. Separate analysis were performed for loblolly and slash pine. Loblolly tests planted in the fall were somewhat larger than those planted in the spring (Table 3). Slash tests showed a similar trend, although statistical significance was not reached for any of the variables. There also appeared a trend for first-year survival to be higher for fall tests, but again, no statistical differences were noted.

Table 3. Average survival and growth for loblolly and slash pine tests established in either spring or fall.

Variable	-----Loblolly-----			-----Slash-----		
	Fall	Spring	Sig. ¹	Fall	Spring	Sig.
1-year survival (%)	92.5	82.7	NS	91.1	87.5	NS
5-year height (m.)	4.2	3.2	*	4.2	3.2	NS
5-year diameter (cm.)	6.2	3.9	*	6.4	4.6	NS
5-year volume (cm. dm.)	4.8	1.4	*	4.7	2.1	NS

¹ Denotes statistical significance. NS indicates non-significant at .05 level of probability. * indicates significant at .05 level of probability.

Performance of common checklots

To alleviate confounding encountered by using tests of differing genetic material, plantings which had checklots in common were selected for further analysis. Four loblolly plantings and six slash plantings met these criteria. Only performance of the checklots in these tests was considered with all other families ignored. Analysis used plot means as input data. Three loblolly checklots (North of Sabine, South of 190 and North Louisiana) and four slash checklots (three early testing lots and a Western Gulf Forest Tree Improvement Program source) were included. The majority of the checklots were from bulked wild collections and did not have a genetic identification other than geographic origin. The three slash early testing lots were bulked seeds of families which had passed various stages of an early testing program. Tables 4 and 5 present the results for loblolly and slash, respectively.

Table 4. Results of analysis for five-year performance of containerized loblolly pine tests planted in spring or fall, using common checklots.

Source	df	5-Year Height			5-Year Diameter			5-Year Volume		
		(m.)			(cm.)			(cu. dm.)		
		M.S.	F		M.S.	F		M.S.	F	
Time (T)	1	24.92	1.96	NS ¹	105.66	1.45	NS	149.52	2.49	NS
Planting (P) within Time	3	12.69	43.75	**	72.86	72.14	**	60.14	18.50	**
Checklot (C)	2	.86	2.96	NS	3.10	3.07	NS	.56	.17	NS
C x T	2	.52	1.79	NS	2.36	2.34	NS	3.70	1.13	NS
C x P	6	.29	1.38	NS	1.01	1.02	NS	3.25	2.02	NS
Error	171	.21			.99			1.61		

¹ Indicates statistical significance. NS indicates non-significant at .05 level of probability. ** indicates significant at .01 level of probability.

Table 5. Results of analysis for five-year performance of containerized slash pine tests planted in spring or fall, using common checklots.

Source	df	5-Year Height (m.)			5-Year Diameter (cm.)			5-Year Volume (cu. dm.)		
		M.S.	F		M.S.	F		M.S.	F	
Time (T)	1	.07	.01	NS ¹	1.00	.03	NS	.33	.01	NS
Planting (P) within Time	4	5.48	22.83	**	31.64	56.50	**	74.01	31.49	**
Checklot (C)	3	.16	.67	NS	.39	.69	NS	3.10	1.31	NS
C x T	3	.07	.29	NS	.55	.98	NS	.86	.36	NS
C x P	10	.24	1.04	NS	1.56	.68	NS	2.35	1.11	NS
Error	221	.23			.82			2.12		

¹ Indicates statistical significance. NS indicates non-significant at .05 level of probability. ** indicates significant at .01 level of probability.

Both of these analyses suggest that time of planting did not have a significant effect on five-year growth for the eleven tests evaluated. While not presented in the table, first-year survival showed similar results. For both species, differences among the individual plantings were highly significant. Thus, location of planting was much more important than time of planting. Additionally, the lack of checklot x time and checklot x planting interactions indicate that these sources performed rather consistently for the two planting times as well as across the various sites.

Comparison of two slash tests

Two of the slash tests were determined to be suitable for a more direct comparison of both planting time and family x time interaction. These tests (#243 and #244) are both located at the Siecke site in Newton County, Texas. Additionally the two tests have the majority of families in common. Site differences between the two planting are considered minimal. Both of these tests consist of trees from open-pollinated seeds collected from slash plantings in east Texas. Test #243 was outplanted in spring, 1987 and test #244 was planted in fall, 1987. For analysis purposes, families not in common in both tests were not used, which subsequently allowed for 25 families to be included. Input data consisted of plot means. Results of the analysis for five-year data are presented in Table 6. Analysis for survival was also performed but is not presented.

A statistically significant difference is observed for time of planting for five-year data. In a contrast to previous results, the spring planted test (#243) performed better than did the fall test (#244). For example five-year average height was 3.9 m. vs 3.6 m for the spring and fall tests, respectively. Both diameter and volume followed similar trends. While family differences are expected, the lack of a family x time interaction suggests very little change in family rankings across the two tests. No differences in first-year survival were noted as both tests averaged above 96 percent.

Table 6. Results of analysis for five-year performance of containerized slash pine tests #243 (spring) and #244 (fall) planted at the Siecke site.

Source	df	5-Year Height (m.)			5-Year Diameter (cm)			5-Year Volume (cu. dm.)	
		M.S.	F		M.S.	F		M.S.	F
Time (T)	1	14.36	55.23	** ¹	80.44	96.92	**	77.13	47.03 **
Family (F)	24	.54	2.05	**	1.02	1.24	NS	3.48	2.13 **
F x T	24	.24	.94	NS	.92	1.12	NS	2.11	1.29 NS
Error	460	.26			.83			1.64	

¹ Indicates statistical significance. NS indicates non-significant at .05 level of probability. ** indicates significant at .01 level of probability.

SUMMARY

The results of this study do not preclude the possibility that containerized progeny tests planted in the spring may suffer from a delayed development for as much as five years after planting. In one analysis, this effect was found to occur in loblolly plantings, and a trend for slash pine to respond similarly was noted. However, separate analyses using only common checklots in plantings of both species showed no effects for time of planting, but showed large differences among planting locations. When two slash pine tests containing the same material were compared, the spring planted test outperformed the fall test. In all analyses, effect of year of planting could not be evaluated.

Use of both common checklots and a slash comparison analysis with the same families showed no source x planting time interaction. This result is somewhat comforting relative to the efficiency of selection in these tests, as family ranks are expected to be fairly consistent for the two planting times.

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VOLUME AND WOOD DENSITY RESULTS
FOR PINUS TECUNUMANII AT EIGHT YEARS OF AGE
IN COLOMBIA

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Abstract. --A broad genetic base of Pinus tecunumanii (Schw.) Equiluz et Perry from high and low elevation populations in Mexico and Central America has been established in Colombia by Smurfit Cartón de Colombia. The species has exhibited good growth in pilot plantings in Colombia and compares favorably with other pines established on a commercial scale in the region. However, P. tecunumanii's susceptibility to stem breakage and poor seed production may limit its potential. Four provenance/progeny tests of P. tecunumanii, that included 12 provenances and 122 half-sib families from Mexico and Central America, were established at San Jose, Colombia (2°30'N latitude) and were assessed at 8 years of age for volume and wood density. Results indicated that provenances and families differed significantly for individual tree volume ($p < 0.01$) but not for wood density. Trees from the Mexican provenances showed greater volume per tree (overbark) than the Central American provenances (0.257 m^3 versus 0.245 m^3). Density per tree was higher for the Central American provenances than the Mexican sources (358 kg/m^3 versus 330 kg/m^3). Montebello (Mexico) and San Jeronimo (Guatemala) were the best provenances in growth and also ranked high for wood density. More than 40 trees from 31 families of P. tecunumanii were selected in the four trials using a combined family and within family selection index. The mean volume for the selected trees was 0.520 m^3 versus 0.252 m^3 for the population. The estimated genetic gain in volume from family and within family selection was 32%.

Keywords: Pinus tecunumanii, provenances, genetic gain

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INTRODUCTION

In the 1980s, Smurfit Cartón de Colombia (SCC) established a number of provenance/progeny trials of tropical and subtropical pines. The seed was mainly obtained from the Central America and Mexico Coniferous Resources Cooperative (CAMCORE), North Carolina State University, through mother tree seed collections in Guatemala, Honduras and Mexico. One of the species most widely tested at the provenance and family level by SCC is Pinus tecunumanii (Schw.) Equiluz et Perry.

In its native range, Pinus tecunumanii occurs from 450 to 2700 m elevation, in a series of small, disjunct populations from Mexico to northern Nicaragua (Dvorak et al 1989). Populations of P. tecunumanii have been classified according to altitude as high elevation (>1500 m) and low elevation provenances (<1500 m) (Dvorak 1986). Recently, phylogenetic analysis using RAPD markers showed genetic differentiation at the DNA sequence level between the high and low elevation ecotypes of P. tecunumanii (Grattapaglia et al 1993). As a member of CAMCORE, SCC has planted 20 genetic trials with approximately 700 half-sib families from 28 provenances of both elevational types.

Results from P. tecunumanii trials planted between 1400 m and 2200 m elevation in Colombia indicate that it has better growth potential than traditionally planted species like P. patula Schiede & Deppe and P. oocarpa Schiede (Ladrach 1986; Dvorak et al 1989; Wright 1992; Wright and Osorio 1992). However, P. tecunumanii's susceptibility to wind damage (Dvorak et al 1993) and its poor seed production in Colombia (Dvorak and Lambeth 1993), could limit the species potential for large scale commercial plantings. Pulp and papermaking wood properties of trees from low elevation provenances of P. tecunumanii have been researched and documented (Dvorak and Kellison 1991; Wright 1987; Palmer and Gibbs 1976). However, little information on wood properties are available for trees from the high elevation provenances.

This paper presents results on the growth (volume) and wood density of P. tecunumanii from high elevation provenances assessed at eight years of age in four studies in Colombia. The limitations of P. tecunumanii are discussed in terms of their implication for further species development and breeding.

MATERIALS AND METHODS

In 1984, four provenance/progeny tests of P. tecunumanii were

established on one site at San Jose, Department of Cauca, Colombia. The trial site was located at an altitude of 1750 m with mean annual rainfall of 2060 mm. Tests 02E and 06A included sources of P. tecunumanii from southern Mexico, tests 16B and 11C contained provenances from Central America. One hundred and twenty-two half-sib families were represented in all four tests. A common P. tecunumanii control, made up of a mixture of seed from selected trees from San Jeronimo, Guatemala (#200) was included in all four sets. The P. oocarpa check lot (#206) from a seed stand in Brazil was included in the Central American tests. The experimental design was a randomized complete block with nine replications and six tree family row plots. The spacing was 3.0 m x 2.5 m and all seedlings were fertilized at the time of planting. The climatic and geographic data for the test sites are presented in table 1.

Eight year assessments were made on total tree height (nearest 0.5 m), outside bark diameter (dbh to the nearest 0.5 cm), and stem straightness and branch diameter (1 to 3 scale with 3 being the best). Assessments were made for stem breakage, foxtail and forks and scored as a "yes" or "no". Wood samples were taken from a total of 27 trees per family from sets 02E (Mexico) and 11C (Central America) using an 8 mm increment borer at 1.3 m stem height. Overbark volume for juvenile trees was calculated using an equation developed by Ladrach (1986): $Vol (m^3) = 0.00003D^2H$. Density was determined using green volume and dry weight. Waller-Duncan tests were used to detect differences among provenance means. Data analyses were done using SAS (SAS, 1992).

Table 1. Provenances and control lots of Pinus tecunumanii included in four genetic tests at San Jose farm, Colombia.

Provenances	Country	Latitude	Elevation (m)	Annual Rainfall (mm)
Jitotol	Mexico	17°02'N	1705	1701
Chempil	Mexico	16°45'N	2120	1146
San José	Mexico	16°42'N	2322	1252
Las Piedrecitas	Mexico	16°22'N	2430	1252
Montebello	Mexico	16°06'N	1705	1909
San Lorenzo	Guatemala	15°05'N	2000	1700
San Vicente	Guatemala	15°05'N	1945	1700
San Jeronimo	Guatemala	15°03'N	1735	1200
Km 47	Guatemala	14°35'N	2100	1543
La Soledad	Guatemala	14°31'N	2427	1543
Celaque	Honduras	14°33'N	1785	1273
Las Trancas	Honduras	14°07'N	2130	1579
Control Lot 200: <u>P. tecunumanii</u> - San Jeronimo (Guatemala)				
Control Lot 206: <u>P. oocarpa</u> - Agudos (Brazil)				

Individual and within family heritabilities were calculated in the standard way using methods described by Becker (1986). Family heritability was calculated using an adjusted estimate of additive variance proposed by Finney (1956), in Shelbourne (1969). The adjustment takes into account the effect of making selections in native stands of small population size. The adjusted family heritabilities h^2_{fa} are a more conservative estimate than values obtained in the normal way. Family and within family selections were made in the field using an index developed by the CAMCORE Cooperative: $I = h^2(f) P(f) + h^2(w) P(w)$ where I was the index value, h^2_{fa} the adjusted family heritability, $P(f)$ the family deviation from the test mean, $h^2(w)$ the within-family heritability, and $P(w)$ the deviation of the individual from the family mean. The selection index was based on volume. Those trees not meeting the threshold values for stem straightness and branch diameter or had defects were rejected (Balocchi 1990). Estimates of genetic gain from family and within family selection were calculated in the normal way (Shelbourne 1969). Selection intensity for families and trees within families was 1.266 and 2.286, respectively. Genetic correlations for height and volume assessed at age 5 and age 8 were obtained using formulae described by Becker (1986).

RESULTS AND DISCUSSION

The analysis of variance indicated significant differences ($p < 0.01$) in mean tree volume among provenances in sets 02E (Mexico) and 11C (Central America), but not for those in sets 06A and 16B. No differences in density were found among provenances in the sampled sets, but families within provenances were statistically different ($p < 0.05$) in every test for individual tree volume and wood density.

On the average the Mexican provenances, sets 06A and 02E, showed slightly greater tree volume (0.257 m^3) than the Central American provenances, (0.245 m^3), sets 16B and 11C (table 2), but wood density was higher for the Central American provenances (tables 3 and 4).

Table 2. Mean volume for P. tecunumanii at eight years of San José, Colombia.

Test No.	No. Families	No. Tree	Survival (%)	Mean Height (m)	C.V. (%)	Mean DBH (cm)	Tree Volume (m^3)
06A	28	1312	87	16.8	17.3	22.3	0.272
02E	39	1819	87	15.9	23.6	21.4	0.247
16B	41	1930	88	17.0	16.2	21.4	0.256
11C	34	1743	88	15.6	17.9	21.4	0.233

Table 3. Individual tree volume and wood density for provenances of Pinus tecunumanii from Central America at San José, Colombia.

Provenances	Test 11C		Test 16B
	Tree Volume ^a (m ³)	Density ^b (Kg/m ³)	Tree Volume ^b (m ³)
Control 200	0.289 a	373	0.304
San Jeronimo	0.284 a	364	0.277
San Vicente	0.216 b	360	0.262
Control 206	0.226 b	367	0.261
Km 47	----	---	0.256
San Lorenzo	0.215 b	367	0.255
Celaque	0.223 b	360	0.252
Las Trancas	0.202 b	341	0.234
La Soledad	0.232 b	345	----
Test Mean ^c	0.233	358	0.256

^a Different letters mean significance at the probability level of 0.05

^b No significant differences among provenances

^c Does not include control lots.

Table 4. Individual tree volume and density for provenances of Pinus tecunumanii from Mexico at San José, Colombia.

Provenances	Test 02E		Test 06A
	Tree Volume ^a (m ³)	Density ^b (kg/m ³)	Tree Volume ^a (m ³)
Control 200	0.313 a	350	0.294
Montebello	0.290 ab	337	0.288
Jitotol	0.238 c	331	0.280
Chempil	0.263 bc	329	0.275
Las Piedrecitas	0.239 c	329	0.245
San José	0.204 d	323	----
Test Mean ^c	0.247	330	0.272

^a Different letters mean significance at the probability level of 0.05

^b No significant differences among provenances

^c Does not include control lots

The mean tree volume for the high elevation provenances from each test ranged from 0.233 to 0.272 m³ and compared favorably with values found for the low elevation provenance of Mountain Pine Ridge, Belize, and San Rafael del Norte and Yucul, Nicaragua (0.207 to 0.281 m³) planted in other tests in the same regions of Colombia (Wright 1992; Wright and Osorio 1992).

At an altitude of 2400 m in Colombia, the high elevation provenances of San Jeronimo and San Lorenzo, Guatemala, were 45% and 55% less productive, respectively, in mean tree volume than at 1750 m altitude. Such interactions were also found by Dvorak et al (1989).

The best high elevation provenances for individual tree volume also ranked at the top in density. Wood density estimates were lower for the Mexican and Central American provenances than for the low elevation provenance of Mountain Pine Ridge, Belize (Wright and Osorio, 1992).

Volume productivity was adversely affected by stem breakage with large increases between ages 5 and 8 for tests 02E and 11C (table 5). Preliminary results suggest a direct relationship between stem breakage percent and branch size (Dvorak et al 1993). The authors also found the trait of stem breakage was under moderate genetic control and improvement should be possible through selection and breeding.

Table 5. Mean stem breakage (%) at 5 and 8 years of P. tecunumanii from Central America and Mexican provenances in four tests in Colombia.

Test No.	Stem Breakage (%)		Provenance Range (8 years)
	Mean (5 years)	Mean (8 years)	
02E	18	32	23-38
06A	20	27	19-37
16B	12	15	12-20
11C	13	38	30-45

Genetic correlations between total height at 5 and 8 years were moderate (range 0.60 - 0.71) in all sets except 06A which was extremely low (0.17). Volume correlations between ages 5 and 8 were also moderate (0.57 - 0.79). Individual tree heritability (h^2_1) decreased from age 5 to 8 (table 6). Results suggest that the best time to select trees of P. tecunumanii to maximize genetic gain may be in younger rather than an older genetic tests.

Forty-one trees in 31 families were selected in the four trials using the selection index. The mean individual tree volume for the selected trees was 0.520 m³ versus the test mean of 0.252 m³. Estimated gain for volume over the population mean was 32%. The 31 selections made in the four provenance/progeny tests of P. tecunumanii compliment those made previously in 8 years old tests in Colombia.

Table 6. Individual tree (h^2_i), family (h^2_{fa}) and within family (h^2_w) heritabilities for P. tecunumanii at 5 and 8 years in four tests in Colombia.

Test No.	5 years			8 years		
	h^2_i	h^2_{fa}	h^2_w	h^2_i	h^2_{fa}	h^2_w
06A	0.21	0.57	0.10	0.12	0.51	0.09
02E	0.44	0.50	0.30	0.31	0.58	0.25
16B	0.31	0.55	0.24	0.11	0.56	0.08
11C	0.47	0.49	0.29	0.35	0.57	0.31

Despite the potential for good genetic gains in volume for P. tecunumanii, the large scale use of this species in Colombia is jeopardized by the fact that it is a poor seed producer. Results from a survey on P. tecunumanii conducted throughout the tropics and subtropics showed that seed production for the species was poor close to the equator (<8 filled seeds/cone), but was reasonable (20-40 filled seeds/cone) between 17° and 28°S latitude, (Dvorak and Lambeth 1993). Trees from high elevation provenances appeared to be poorer producers than those from low elevation provenances. Commercial seed supplies of P. tecunumanii from Central America and Mexico appear uncertain in the future because of the wide-spread cutting of pine forests in the region and poor funding levels for national seed banks. The San Jeronimo provenance, which is one of the best sources for Colombia, has been reduced to 50% of its original size in Guatemala since 1980, (Dvorak and Donahue 1992).

CONCLUSIONS

Pinus tecunumanii has great growth potential in Colombia. Heritabilities were high for volume and good gains can be made through family and within family selection. The wood density of the species seems acceptable, but more information is needed on the pulping and paper making qualities of trees from the high elevation provenances. Top stem breakage averaged 28% at eight years in the four trials at San Jose. This, combined with poor seed production, may reduce the potential value of P. tecunumanii in Colombia. Alternate strategies to minimize these local limitations include the establishment of P. tecunumanii plantings in areas protected from strong seasonal winds and to locate seed orchards in countries where seed production is more rapid, abundant and consistent from year to year.

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SESSION 8

Molecular Genetics

cDNA CLONING OF WATER DEFICIT-INDUCIBLE GENES FROM LOBLOLLY PINE

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Abstract.--Pine trees produce new proteins when growing under water deficit and this is achieved principally by activating formerly quiescent genes. Determining the identity of these genes, how they sense changes in the environment and the role their proteins play in drought tolerance is important for an appreciation of tree growth under stress and for emerging biotechnologies. We have isolated cDNA clones (DNA copies of the mRNA) of drought-induced genes from Loblolly Pine (Pinus taeda). DNA sequencing and computer aided comparisons reveal strong similarities between these pine clones and stress-induced genes in other plants, particularly those involved in lignin synthesis.

Keywords: gene expression, drought, cDNA cloning, Pinus taeda L., stress proteins, O-methyl transferase, SAM synthetase, ABA.

INTRODUCTION

Drought is one of the principal environmental stresses affecting tree growth. Whether episodic or perennial, drought impairs development and productivity and renders trees susceptible to secondary, normally, sub-lethal insults. In extreme cases drought will destroy large areas of forest thus contributing to land erosion and to desertification. Drought stress is the most common cause of pine seedling mortality in both naturally regenerated and planted stands in the U.S. (Anonymous 1986). In one long term study drought accounted for 57% of first year seedling mortality (Williston 1972)

The physiological effects of water shortage upon trees have been investigated extensively (Pallardy 1981, Newton et al. 1985,1990, Valluri et al. 1988, Vance and Zaeer 1988) and the development of drought-tolerant, high-performance trees has been pursued through breeding programs (Wakeley 1954, van Buijtenen et al. 1974, Newton et al. 1986). Currently these approaches are being supplemented by the molecular techniques of genome mapping to assist in identifying traits with particular chromosomal fragments (Neale et al. 1989, Tauer et al. 1992). As classical and modern approaches converge there is a growing need for information on the molecular effects of environmental stress on trees but little information is available at present.

By contrast the molecular biology of drought in herbaceous plants is an active field. Alteration in gene expression in plants growing under water-deficit has been observed for many crop species (Heikkila et al. 1984, Ramagopal 1987, Guerrero and Mullet 1988) and the isolation of clones from

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cDNA libraries, using altered expression as a selection criterion, has permitted the identification of many stress-inducible proteins (Skriver and Mundy 1990, Bray 1991, Olsen et al. 1992, Bohnert et al. 1992). In some cases functions have been ascribed to the putative polypeptides bases on sequence similarities to previously characterized proteins (Singh et al. 1989, Borkird et al. 1990, Downing et al. 1992). A number of these drought-inducible genes show homology to genes expressed in maturing embryos (Dure et al. 1981, Baker et al. 1988, Dure et al. 1989). Conversely, many of the Late Embryogenesis Abundant (Lea) proteins (Galau et al. 1986, Baker et al. 1988) are induced in the vegetative tissue of mature plants during periods of dessication or by exogenous application of ABA (Gomez et al. 1988, Mundy and Chua 1989, Close et al. 1989, Bray 1990). No function has been elucidated for the majority of the water deficit-inducible proteins, however, the strong sequence conservation among species has prompted speculation as to their role in dessication tolerance (Dure et al. 1989, Dure 1993).

Work from our laboratories has shown that dehydration does alter the pattern of protein synthesis in loblolly pine (Valluri et al. 1988, 1989; Vance et al. 1988; Funkhouser et al. 1993), an expected result and one which is consistent with other plants. We have sought to identify these proteins through cDNA cloning. Here we report the construction of a cDNA library from roots of a drought-stressed loblolly pine, selection and tentative identification of genes. A number of clones with striking sequence similarity to wound/pathogen-induced proteins have been recognized, however, most of the pine clones have structural features or patterns of expression which distinguish them from their homologs.

METHODS

Plant Growth and Water Status

Plants were acclimatized by irrigating daily with Reverse Osmosis (RO) water for seven days. Water was then withheld, in staggered fashion, so that different sets of plants were deprived for various periods. Control plants were watered continuously. All plants were harvested pre-dawn on the same day. Plant pre-dawn water potential was determined with a pressure bomb (Scholander et al. 1964) and thermocouple psychrometry with methods described previously (Emadian and Newton 1989; Castro-Jimenez et al. 1989; Newton et al. 1989). A branch from each plant was used for measurement, the rest of the plant was harvested, leaves, stem and roots were frozen separately.

RNA Isolation

Conventional methods of RNA preparation are often inefficient when applied to pine due to the high levels of resins and phenolics in pine tissue, especially needles. We have developed a method for preparing RNA which is free from impurities and suitable for enzymatic manipulation (Chang et al. 1993). This method was used in this study.

cDNA Library Construction and Molecular Methods

The root tissue of an 8 month old seedling with a water potential of -1.1 MPa was chosen for cDNA library construction. Poly(A)⁺ RNA was isolate

from total RNA using the PolyA Tract magnetic sphere system (Promega, WI). A cDNA library was prepared using the Stratagene 1-ZAP cDNA Synthesis kit according to manufacturer's recommendations (Stratagene, CA). Standard molecular methods were used in other cases (Sambrook et al. 1989)

DNA Sequencing and Data Analysis

DNA was sequenced by the Sanger method using Sequenase™ (USB, Cleveland, OH) in the DNA Technologies Laboratory, Texas A&M University. Computer searches of the NIH Genbank and Swiss Protein databank were performed. Data was analyzed using the DNA Star program (Madison, WI).

RESULTS AND DISCUSSION

Isolation and Expression of the Drought-Regulated Genes

A cDNA library of 7.2×10^6 pfu was constructed from poly A(+) RNA isolated from the roots of a 5 month old, water-stressed pine seedling. Clones of drought stress-regulated genes were isolated by differential screening of about 15,000 independent plaques essentially as described by Adair et al. (1992). From 28 putative water stress-responsive clones identified in a primary screening, 15 were confirmed by Northern analysis. Six distinct clones which contained cDNA molecules whose size was similar to that of the mRNA to which they hybridized, were sequenced.

The gene corresponding to clone LP1 is induced by water deficit in both needles and roots. In stems, however, the expression appears to be constitutive (data not shown). In needles, the expression drops sharply as water stress increases; in roots, this diminution is gradual. The sequence and transcript size predict a 5' untranslated region of around 300 nucleotides for this mRNA (data not shown). LP1 has a long open reading frame beginning with an ATG which could encode a polypeptide of 224 amino acids. The predicted sequence of this protein bears strong resemblance (40% identity, 62% similarity) to a Caffeoyl-CoA-3-O-Methyltransferase from parsley (Fig. 1, Schmitt et al. 1991). In parsley, the mRNA for Caffeoyl-CoA-3-O-Methyltransferase has a 5' untranslated region of about 300 nucleotides (Schmitt et al. 1991). The parsley protein has been implicated in plant defense responses to pathogens and is part of the phenylpropanoid pathway (Pakusch et al. 1989, 1991, Hahlbrock and Scheel 1989). Caffeoyl-CoA-3-O-Methyltransferase converts Caffeoyl-CoA to Feruloyl-CoA, a precursor of lignin (Lewis and Yamamoto 1990, Sederoff and Chang 1991). In parsley, this enzyme is induced by fungal elicitors and is implicated in the synthesis of ferulic esters for cell wall reinforcement, a defense response to pathogen attack (Pakusch et al. 1989, 1991). Evidence for this pathway in pine has not yet been furnished. In parsley cell culture, induced expression is rapid and transient (Schmitt et al. 1991), in loblolly pine seedlings subjected to water stress a more sustained induction is apparent (data not shown).

1M E S I Y K Y M L E T F T R H R E D E A S	LP1
1	M A S N G E S K H S E V G H K S L L Q S D A L Y Q Y I L E T S V Y P R E P E A M	PARSELEY
23	K E L W E - - - R T W N L T Q R G E M M T L P D Q V Q F L R L M V K M S G A K	LP1
41	K E L R E V T A K H P W N L - - - - M T T S A D E G Q F L N M L L K L I N A K	PARSELEY
59	K A L E I G V F T G Y S L L N I A L A L P S D G K V V A V D P G D D P - K F G W	LP1
76	N T M E I G V Y T G Y S L L A T A L A L P D D G K I L A M D I N R E N Y E I G L	PARSELEY
98	P C F V K A G V A D K V E I K K T T G L D Y L D S L I Q K G E - K D C F D F A F	LP1
116	P I I E K A G V G H K I D F R E G P A L P V L D H M L E D G K Y H G T F D F V F	PARSELEY
137	V D A D K V N Y V N Y H P R L M K L V R V G G V I I Y D D T L W F G L V G G K D	LP1
156	V D A D K D N Y I N Y H K R L I D L V K I G G L I G Y D N T L W N G S V A - - Q	PARSELEY
177	P H N L L K N D Y M R T S L E G I K A I N S M V A N D P N L E V A T V F M G Y G	LP1
194	P A D A P M R K Y V R Y Y R D F V I E L N K A L A A D P R I E I C M L P V G D G	PARSELEY
217	V T V C Y R T A	LP1
234	V T L C R R I S	PARSELEY

Figure 1. Amino acid homology between predicted polypeptides from loblolly pine clone LP1 and parsley Caffeoyl-CoA-3-O-Methyltransferase (Schmitt et al. 1991). Solid lines indicated identical residues, dotted lines, similar residues.

A number of cDNA clones of putative O-Methyltransferases have been isolated recently. Those from parsley, alfalfa and aspen were described as biphasic Caffeic Acid O-Methyltransferases capable of converting both caffeic acid to ferulic acid and subsequently of methylating 5-hydroxy ferulic acid to form sinapic acid (Bugos et al. 1991, Gowri et al. 1991). An O-Methyltransferase from Ice plant has been shown to convert myo-inositol to ononitol, a precursor of pinitol, a cyclic sugar with osmoremedial properties (Vernon and Bohnert 1992). When all five predicted polypeptides are aligned (Fig. 2) we see a striking dichotomy; parsley and pine forming one class of sequence while alfalfa, aspen and ice plant form the second class, with little homology between the groups. Within the second group we have enzymes which have quite different substrate specificities thus the sequence similarity of the alfalfa and aspen enzymes to the OMT from ice plant is most notable. This feature may reflect the broader substrate specificity of the "biphasic" enzymes. It would be of great interest to determine the ability of the alfalfa and aspen enzymes to methylate myoinositol.

LP2 is water deficit-inducible in all tissues with maximal expression occurring in the stems. Expression remains high in all tissues even under severe stress (approximately 30 days without water). The putative protein encoded by this clone is virtually identical to S-adenosylmethionine synthetase from a number of plant species (Fig. 3, Peleman et al. 1989, Larsen and Woodson 1991, Kawalleck et al. 1992).

S-adenosylmethionine synthetase (SAM synthetase) catalyses the biosynthesis of S-adenosylmethionine from methionine and ATP (Tabor and Tabor 1984). S-adenosylmethionine is a co-factor in numerous biochemical reactions acting

1	M	-	-	-	-	E	T	F	L	F	T	S	E	S	V	N	E	G	H	P	D	K	L	C	D	Q	I	S	D	A	V	L	D	A	C	L	T	Q	D	P	LP2	
1	M	-	-	-	-	E	T	F	L	F	T	S	E	S	V	N	E	G	H	P	D	K	L	C	D	Q	I	S	D	A	V	L	D	A	C	L	E	Q	D	P	ARABIDOPSIS 1	
1	M	-	-	-	-	E	T	F	L	F	T	S	E	S	V	N	E	G	H	P	D	K	L	C	D	Q	I	S	D	A	V	L	D	A	C	L	E	Q	D	P	ARABIDOPSIS 2	
1	M	A	A	A	A	D	T	F	L	F	T	S	E	S	V	N	E	G	H	P	D	K	L	C	D	Q	I	S	D	A	V	L	D	A	C	L	A	Q	D	A	CARNATION	
1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	PARSELEY			
37	D	S	K	V	A	C	E	T	C	T	K	T	N	M	V	M	V	F	G	E	I	T	T	K	A	D	V	D	Y	E	Q	I	V	R	K	T	C	R	E	I	LP2	
37	D	S	K	V	A	C	E	T	C	T	K	T	N	M	V	M	V	F	G	E	I	T	T	K	A	T	I	D	Y	E	K	I	V	R	D	T	C	R	S	I	ARABIDOPSIS 1	
37	D	S	K	V	A	C	E	T	C	T	K	T	N	M	V	M	V	F	G	E	I	T	T	K	A	T	V	D	Y	E	K	I	V	R	D	T	C	R	A	I	ARABIDOPSIS 2	
41	E	S	K	V	A	C	E	T	C	T	K	T	N	L	V	M	V	F	G	E	I	T	T	K	A	N	V	D	Y	E	K	I	V	A	D	T	C	R	E	I	CARNATION	
1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	PARSELEY			
77	G	F	I	S	D	D	V	G	L	D	A	D	H	C	K	V	L	V	N	I	E	Q	Q	S	P	D	I	A	Q	G	V	H	G	H	F	T	K	R	P	E	LP2	
77	G	F	I	S	D	D	V	G	L	D	A	D	K	C	K	V	L	V	N	I	E	Q	Q	S	P	D	I	A	Q	G	V	H	G	H	F	T	K	R	P	E	ARABIDOPSIS 1	
77	G	F	V	S	D	D	V	G	L	D	A	D	K	C	K	V	L	V	N	I	E	Q	Q	S	P	D	I	A	Q	G	V	H	G	H	F	T	K	C	P	E	ARABIDOPSIS 2	
81	G	F	V	S	P	D	V	G	L	D	A	D	N	C	K	V	L	V	Y	I	E	Q	Q	S	P	D	I	A	Q	G	V	H	G	H	L	T	K	R	P	E	CARNATION	
1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	PARSELEY			
117	E	I	G	A	G	D	Q	G	H	M	F	G	Y	A	T	D	E	T	P	E	L	M	P	L	T	H	V	L	A	T	K	L	G	A	K	L	T	E	V	R	LP2	
117	D	I	G	A	G	D	Q	G	H	M	F	G	Y	A	T	D	E	T	P	E	L	M	P	L	S	H	V	L	A	T	K	I	G	A	R	L	T	E	V	R	ARABIDOPSIS 1	
117	D	I	G	A	G	D	Q	G	H	M	F	G	Y	A	T	D	E	T	P	E	L	M	P	L	S	H	V	L	A	T	K	L	G	A	R	L	T	E	V	R	ARABIDOPSIS 2	
121	D	I	G	A	G	D	Q	G	H	M	F	G	Y	A	T	D	E	T	P	E	L	M	P	L	S	H	V	L	A	T	K	L	G	A	R	L	T	E	V	R	CARNATION	
1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	PARSELEY			
157	K	N	G	T	C	P	W	L	R	P	D	G	K	T	Q	V	T	I	E	Y	R	N	E	G	G	A	M	V	P	E	R	V	H	T	V	L	I	S	T	Q	LP2	
157	K	N	G	T	C	R	W	L	R	P	D	G	K	T	Q	V	T	V	E	Y	Y	N	D	N	G	A	M	V	P	V	R	V	H	T	V	L	I	S	T	Q	ARABIDOPSIS 1	
157	K	N	G	T	C	A	W	L	R	P	D	G	K	T	Q	V	T	V	E	Y	Y	N	D	K	G	A	M	V	P	I	R	V	H	T	V	L	I	S	T	Q	ARABIDOPSIS 2	
161	K	N	G	T	C	A	W	L	R	P	D	G	K	T	Q	V	T	V	E	Y	Y	N	E	N	G	A	M	V	P	I	R	V	H	T	V	L	I	S	T	Q	CARNATION	
1	-	N	G	T	C	A	W	L	R	P	D	G	K	T	Q	V	T	V	E	Y	Q	N	D	H	G	A	M	V	P	I	R	V	H	T	I	L	I	S	T	Q	PARSELEY	
197	H	D	E	T	V	T	N	D	Q	I	A	A	D	L	K	E	H	V	I	K	P	V	I	P	E	K	Y	L	D	E	N	T	I	F	H	L	N	P	S	G	LP2	
197	H	D	E	T	V	T	N	D	E	I	A	R	D	L	K	E	H	V	I	K	P	I	I	P	E	K	Y	L	D	D	K	T	I	F	H	L	N	P	S	G	ARABIDOPSIS 1	
197	H	D	E	T	V	T	N	D	E	I	A	R	D	L	K	E	H	V	I	K	P	V	I	P	E	K	Y	L	D	E	K	T	I	F	H	L	N	P	S	G	ARABIDOPSIS 2	
201	H	D	E	T	V	T	N	D	E	I	A	A	D	L	K	E	H	V	I	K	P	V	I	P	E	K	Y	L	D	E	N	T	I	F	H	L	N	P	S	G	CARNATION	
40	H	D	E	T	V	T	N	D	E	I	A	A	D	L	K	E	H	V	I	K	P	V	I	V	P	E	N	Y	L	D	E	K	T	I	F	H	L	N	P	S	G	PARSELEY
237	R	F	V	I	G	G	P	H	G	D	A	G	L	T	G	R	K	I	I	I	D	T	Y	G	G	W	G	A	H	G	G	G	A	F	S	G	K	D	P	T	LP2	
237	R	F	V	I	G	G	P	H	G	D	A	G	L	T	G	R	K	I	I	I	D	T	Y	G	G	W	G	A	H	G	G	G	A	F	S	G	K	D	P	T	ARABIDOPSIS 1	
237	R	F	V	I	G	G	P	H	G	D	A	G	L	T	G	R	K	I	I	I	D	T	Y	G	G	W	G	A	H	G	G	G	A	F	S	G	K	D	P	T	ARABIDOPSIS 2	
241	R	F	V	I	G	G	P	H	G	D	A	G	L	T	G	R	K	I	I	I	D	T	Y	G	G	W	G	A	H	G	G	G	A	F	S	R	K	D	P	T	CARNATION	
80	R	F	V	I	G	G	P	H	G	D	A	G	L	T	G	R	K	I	I	I	D	T	Y	G	G	W	G	A	H	G	G	G	A	F	S	G	K	D	P	T	PARSELEY	
277	K	V	D	R	S	G	A	Y	I	V	R	Q	A	A	K	S	I	V	A	A	G	L	A	R	R	C	L	V	Q	V	S	Y	A	I	G	V	P	E	P	L	LP2	
277	K	V	D	R	S	G	A	Y	I	V	R	Q	A	A	K	S	V	V	A	N	G	M	A	R	R	A	L	V	Q	V	S	Y	A	I	G	V	P	E	P	L	ARABIDOPSIS 1	
277	K	V	D	R	S	G	A	Y	I	V	R	Q	A	A	K	S	V	V	A	N	G	M	A	R	R	A	L	V	Q	V	S	Y	A	I	G	V	P	E	P	L	ARABIDOPSIS 2	
281	K	V	D	R	S	G	A	Y	I	A	R	Q	A	A	K	S	I	V	A	S	G	L	A	R	R	C	I	V	Q	I	S	Y	A	I	G	V	P	E	P	L	CARNATION	
120	K	V	D	R	S	G	A	Y	I	V	R	Q	A	A	K	S	I	V	A	S	G	L	A	R	R	C	I	V	Q	V	S	Y	A	I	G	V	P	E	P	L	PARSELEY	
317	S	I	F	V	D	S	Y	G	T	G	S	I	P	D	K	E	I	L	E	I	I	K	E	H	F	D	F	R	P	G	M	I	T	I	N	L	D	L	K	R	LP2	
317	S	V	F	V	D	T	Y	G	T	G	L	I	P	D	K	E	I	L	K	I	V	K	E	T	F	D	F	R	P	G	M	M	T	I	N	L	D	L	K	R	ARABIDOPSIS 1	
317	S	V	F	V	D	T	Y	E	T	G	L	I	P	D	K	E	I	L	K	I	V	K	E	S	F	D	F	R	P	G	M	M	T	I	N	L	D	L	K	R	ARABIDOPSIS 2	
321	S	V	F	V	D	T	Y	G	T	G	K	I	H	D	R	E	I	L	K	I	V	K	E	N	F	D	F	R	P	G	M	I	A	I	A	L	D	L	K	K	CARNATION	
160	S	V	F	V	D	T	Y	G	T	G	K	I	P	D	R	E	I	L	K	I	V	K	E	T	F	D	F	R	P	G	M	I	S	I	N	L	D	L	K	R	PARSELEY	
357	G	G	N	G	R	F	Q	K	T	A	A	Y	G	H	F	G	R	D	D	P	D	F	T	W	E	T	V	K	P	L	K	W	E	K	A	Q	A	LP2				
357	G	G	N	G	R	F	Q	K	T	A	A	Y	G	H	F	G	R	D	D	P	D	F	T	W	E	V	V	K	P	L	K	W	D	K	P	Q	A	ARABIDOPSIS 1				
357	G	G	N	G	R	F	L	K	T	A	A	Y	G	H	F	G	R	D	D	P	D	F	T	W	E	V	V	K	P	L	K	W	D	K	P	Q	A	ARABIDOPSIS 2				
361	G	G	N	-	R	Y	L	K	T	A	A	Y	G	H	F	G	R	E	D	P	D	F	T	W	E	A	A	K	T	L	K	W	E	K	P	Q	A	CARNATION				
200	G	G	N	G	R	F	L	K	T	A	A	Y	G	H	F	G	R	E	D	P	D	F	T	W	E	V	V	K	P	L	K	W	E	K	A	PARSELEY						

Figure 3. Protein sequence comparison of LP2 to SAM synthetase proteins from *Arabidopsis* (Peleman et al. 1989a,b), *Carnation* (Larsen and Woodson 1991), and *parsley* (Kawalleck et al. 1992). Identical residues are boxed.

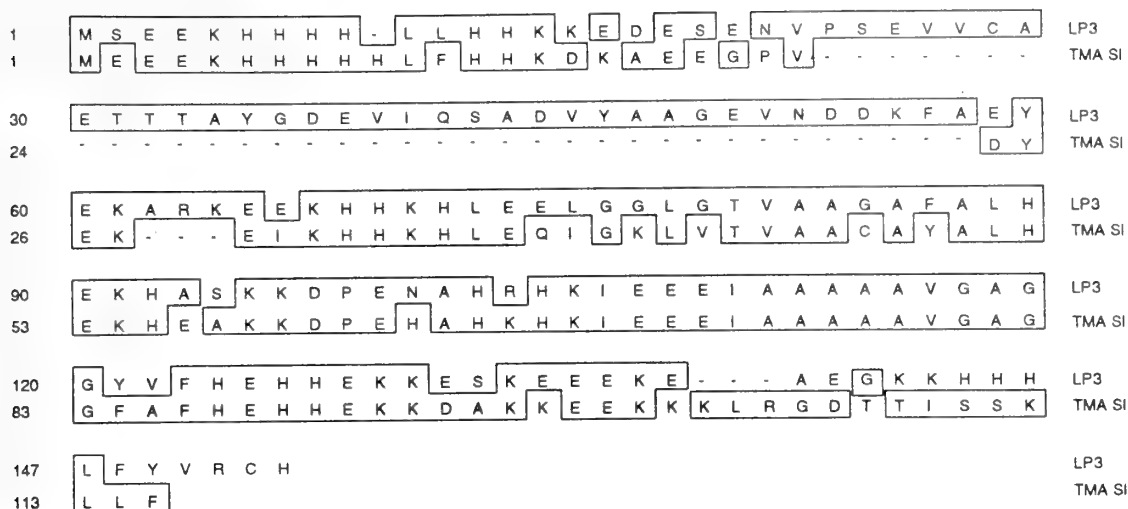


Figure 4. Protein sequence comparison between loblolly pine clone, LP3, and ABA-inducible tomato clone, TMA S1.

as a methyl donor to proteins, lipids, polysaccharides and nucleic acids (Tabor and Tabor 1984), it is also an intermediate in the synthesis of ethylene (Yang and Hoffman 1984). The ubiquity of the enzyme has led to a designation of the SAM synthetase gene as a 'housekeeping gene' but surprisingly the gene shows strong cell specificity being highly expressed in stem, root and callus of *Arabidopsis*, with little expression in leaf (Peleman et al. 1989). Peleman et al. (1989) suggested that SAM synthetase expression was highest in lignifying tissue. Recently, in parsley, induction of SAM synthetase gene expression by fungal elicitors has been demonstrated (Kawalleck et al. 1992). Since lignification is a plant defense response these two observations seem consistent and compatible with our own demonstration of SAM synthetase induction in response to environmental stress. By contrast the requirement for S-adenosylmethionine in ethylene synthesis appears small; SAM synthetase mRNA levels decline markedly in climateric carnation petals (Woodson et al. 1992).

Clone LP3 is expressed predominantly in roots. Levels of mRNA are detected in needles and stems but at much lower levels though the pattern of induction appears similar in all tissues with different water potentials for maximal expression. A polypeptide of 153 amino acids is encoded by LP3 which has 49% identity, 56% similarity to a tomato protein TMA SN1 (Genbank Acc # L08255). This tomato protein is reported to be ABA-inducible and expressed during fruit ripening. Interestingly, the putative loblolly pine protein possesses a region of 34 amino acids (Pro 24 to Ala 58) which is absent from the tomato homolog. If we exclude this region and re-consider only the match the identity rises to 62%.

Levels of the phytohormone ABA rise in plants subjected to water stress (Hartung and Davies 1991) and the effects of water stress on gene expression often can be mimicked by the exogenous application of ABA (Skriver and Mundy 1990). Further, the induction of drought-responsive genes is depressed in mutants deficient in ABA synthesis (Cohen and Bray 1990, Bray 1991). These

observations have led to proposals that ABA acts as the cellular mediator of stress (Zevaart and Creelman 1988) though the observation that some drought-responsive genes are insensitive to ABA suggests the existence of multiple transduction paths and/or additional induction factors (Xu et al. 1990, Bostock and Quatrano 1992).

Concluding Remarks

The cDNA clones described in this paper identify genes induced by water stress in loblolly pine which show strong sequence similarity to genes induced by wounding and pathogen attack in other plants. The putative polypeptides synthesized by loblolly pine have distinctive features. The sequence differences between LP1 and O-methyltransferases from other sources are intriguing (Fig. 2). The substrate specificity of the LP1 protein is currently being determined and this should allow a more accurate definition of the role these proteins play in stress physiology. The induction and sequence of LP2 (SAM synthetase) is consistent with recent findings. The LP3 protein has yet to be defined. These results, combined with our earlier findings on promoter structure and function (Newton et al. 1992, Funkhouser et al. 1993) reveal great similarities at the molecular level between pine and herbaceous plants. These demonstrations indicate a likely success in the expression of pine genes in rapidly regenerating transgenic model plants. Such gene transfer experiments are being conducted in our laboratories and should permit the evaluation of pine gene function for both research and applied purposes.

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CHITINASE GENES AND GENE PRODUCTS IN PINES*

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Abstract. Chitinases are hydrolytic enzymes that are thought to play an important role in plant defense against pathogenic fungi, and potentially in guiding normal plant growth and development. We are investigating chitinase genes and gene products at the nucleic acid and protein levels. At the nucleic acid level, we have previously cloned chitinase sequences from pine using PCR, and used one of the amplification products as a probe to screen a genomic library. Based on preliminary sequence data, the genes are more similar to extracellular chitinases from herbaceous plants than to vacuolar forms. At the protein level, we found that chitosan elicits increases in both extracellular and intracellular chitinase activities in suspension cultured cells. The suspension cultures should prove useful for bulk purification of extracellular and vacuolar chitinases.

INTRODUCTION

We are studying the cellular and molecular basis for defense mechanisms in trees. By gaining insight into these defense processes, we hope to better understand tree-microbe interactions, both symbiotic and pathogenic, at the biochemical level. Chitinase activity serves as a useful marker for studying defense responses, since rapid expression of chitinase genes is often associated with disease resistance (Collinge et al. 1993). It is of interest to note that transgenic plants containing chitinase gene constructs had increased resistance to a chitin-containing root pathogen; in the same plants, the colonization of roots by a chitin-containing mycorrhizal fungus was not diminished (Vierheilig et al. 1993).

The location, and kinetics of accumulation, of chitinase gene products have profound effects on their efficacy as defensive enzymes. Chitinases are encoded by multigene families in trees (Parsons et al. 1989; Davis et al. 1991), and the regulation of individual chitinase genes can occur at many different levels. For example, mRNA and protein levels can be regulated by either synthesis or degradation. The expression of specific genes might occur in particular organs or in specific cell types. The stimulus for turning the gene "on" might be local (near a site of cellular injury) or systemic (distant from a site of injury). The protein, in turn, can be intracellular (targeted to the vacuole) or extracellular. As a step toward characterizing chitinase regulation in pines, we report in this paper that both intracellular and extracellular chitinase accumulation can be detected in pine suspension cultures, and that chitinase activity is stimulated by treatment with chitosan, a component of fungal cell walls.

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MATERIALS AND METHODS

Suspension cultured cells were initiated from callus of a single loblolly pine seedling (family 10-38), and maintained by weekly transfers to fresh medium (Lesney, 1989). The time of transfer to fresh medium was designated "day 0." Chitosan was prepared as previously reported (Lesney, 1989) and then dispensed into the suspension (~50 ug/ml final concentration) on day 1 or day 6. Using this amount of chitosan, elicitation occurred, but little or no cellular death accompanied the treatment (Lesney, 1989). Treatments were replicated three times, with each flask representing a replicate.

Twenty-four hours after chitosan addition (day 2 and day 7), cells and medium of chitosan-treated and untreated cultures were harvested separately. Cells were harvested by filtering cultures through filter paper under gentle vacuum, and were then frozen and ground in liquid nitrogen using a mortar and pestle and resuspended in 3-5 volumes of 50 mM sodium acetate (pH 5.0) containing 1% polyvinylpyrrolidone. After centrifugation (5,000 x g for 10 min), the supernatant was transferred to a fresh tube and used for determination of protein concentration (Brown et al. 1989) and for chitinase activity (Cabib 1988). The culture medium was used directly in the assays.

RESULTS AND DISCUSSION

Chitinase genes were previously cloned from pines using PCR and degenerate oligonucleotide primers (J.D., unpublished data). Partial sequence analysis indicates the pine chitinase genes we cloned are more similar in sequence to extracellular chitinases than to vacuolar chitinases. In addition, the chitinase genes we have cloned appear to belong to a medium-sized gene family (defining the gene family as consisting of members >90% identical in nucleotide sequence). We were interested in testing whether cell suspension cultures would be useful for eliciting chitinase genes, and whether suspensions might provide a convenient source of enzyme material.

For clarity of presentation, chitinase specific activity values (that is, chitinase activity / unit protein / unit time) were normalized to the specific activity present in the untreated cells on day 2 (Table 1). As such, the values represent the relative chitinase activities in the various treatments.

Chitinase activity was observed in both intracellular and extracellular fractions of untreated cultures. This is consistent with the expectation that a certain level of "constitutive" chitinase gene expression occurs in suspension cultures (Esaka et al. 1990). Given the likelihood that chitinase subcellular targeting mechanisms are similar in gymnosperms and angiosperms (Neuhaus et al. 1991), these data indicate that there is a minimum of two chitinase genes, one encoding a vacuolar enzyme and another encoding an extracellular enzyme, that are constitutively expressed in suspension cultures.

Table 1. Relative amount of chitinase activity in chitosan-treated and untreated loblolly pine suspension cultures, normalized to the specific activity of chitinase in day 2 cells. Probability of differences between chitosan treated and untreated suspensions being due to chance alone is indicated (*, $p < .05$; **, $p < .01$; ***, $p < .001$).

DAY 2 CELLS			DAY 7 CELLS		
<u>Control</u>	<u>Chitosan</u>		<u>Control</u>	<u>Chitosan</u>	
1.0	1.3	*	2.9	5.2	**
DAY 2 MEDIUM			DAY 7 MEDIUM		
<u>Control</u>	<u>Chitosan</u>		<u>Control</u>	<u>Chitosan</u>	
1.0	2.1	**	2.2	4.1	***

At day 7, constitutive levels of chitinase in untreated cultures had increased 2- to 3-fold over the levels found at day 2. This could reflect autoelicitation of chitinase activity by carbohydrates secreted into the medium by the cells (Lesney, 1989).

Chitosan treatment induced statistically significant increases of chitinase activity in the intracellular and extracellular compartments on both sample dates. The competence of cells to express chitinase in response to chitosan was similar (1.3 to 2.1-fold) in both day 2 and day 7 cells, but the highest chitinase activities were found in cultures treated with chitosan on day 7. This suggests that day 7 cultures are the most desirable starting materials for bulk purification of both vacuolar and extracellular chitinase, which will be useful for antibody production.

In this paper we have described chitinase induction in loblolly pine cells in response to chitosan, a fungal cell wall component. Short-term objectives include identification of chitinase transcripts in cell suspensions. Longer-term objectives include development of a detailed understanding of the spatial and temporal expression of chitinase genes in specific tree-microbe interactions, and use of genetic transformation technology to experimentally manipulate chitinase gene expression.

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VARIATION OF RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) MARKERS IN PECAN

M. Imbalzano ^{1,2/} and M. Stine ^{1/}

Abstract.—The potential use of RAPD markers to measure intraspecific variation in pecan (*Carya illinoensis* (Wang.) K. Koch) was examined. Three parent/progeny lines were screened with nine Operon® primers to identify RAPD markers and to calculate their inheritance ratios. These markers were then identified in 77 individuals from a provenance test. Based on chi-square analysis, 24% of the markers identified in parent/progeny lines were not inherited in a Mendelian fashion. Of the bands scored, 24% in the inheritance study and 33% in the provenance study were not reproducible. Phylogenetic analyses were conducted on the full data set of markers identified in the provenance test and three subsets of the data. The first subset contained only the heritable and reproducible markers, the second included only the heritable markers, and the third contained only the reproducible markers. For none of the data sets examined does the RAPD data agree with anticipated clustering based on geography, with the exception of a Louisiana and western Mississippi cluster. The potential causes of these results are discussed.

Keywords: *Carya illinoensis* (Wang.) K. Koch, polymerase chain reaction, molecular markers.

INTRODUCTION

The development of the RAPD technique by Williams et al. (1990) and the arbitrarily primed DNA technique by Welsh and McClelland (1990) have raised interest in the application of RAPDs in many areas of research. RAPDs can be less time-consuming and less expensive than other methods, such as restriction fragment-length polymorphisms (RFLPs), which makes it very appealing to many researchers. Studies have demonstrated that RAPD primers can be

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used to identify DNA polymorphisms in a wide variety of animal, plant, and bacterial species (Welsh and McClelland 1990, Williams et al. 1990, Goodwin and Annis 1991, Hu and Quiros 1991).

Many potential applications for this technique are currently being explored. Its usefulness in the determination of taxonomic identity has already been demonstrated. Hu and Quiros (1991) have shown that RAPD markers can differentiate among cultivars of broccoli (*Brassica oleracea*) and among cultivars of cauliflower (*B. oleracea*). Arnold et al. (1991) demonstrated that RAPD primers could produce species-specific markers for irises, which were then used to demonstrate hybridization and introgression. Using six maize inbred lines and five hybrids from the inbreds, Welsh et al. (1991) conducted a double blind experiment that used RAPD markers to identify the parents of the hybrids. RAPD markers may also have potential use in phylogenetic studies. Halward et al. (1992) identified species-specific markers for 29 diploid wild species of peanut (*Arachis hypogaea*). The characters were analyzed by two phylogenetic reconstruction programs, PAUP and HyperRFLP, to determine species relationships. The two programs produced almost identical dendrograms, which were consistent with dendrograms produced with morphological, enzymatic, and RFLP data.

Based on the usefulness of RAPD markers as demonstrated in the literature, we wished to conduct a study on the potential use of RAPD analysis to investigate intraspecific variation in pecan (*Carya illinoensis* (Wang.) K. Koch). To determine if the RAPD characters would be useful in cladistic analysis, the data were used in the phylogenetic reconstruction program, Hennig86 (Farris 1988). A clustering of individuals according to their geographic source was expected.

MATERIALS AND METHODS

Leaf tissue was collected from 77 individuals in a pecan provenance test, located at Idlewild Experimental Station near Clinton, Louisiana. The provenance test contains accessions from 10 states throughout the natural range of pecan. To verify the inheritance of the RAPD markers, the leaf tissue of three pecan pedigrees, each consisting of two parents and 10 progeny, was obtained from the Pecan Genetics and Breeding Station Orchard in Brownwood, Texas.

A modification of Murray and Thompson's (1980) method was used to extract total cellular DNA from the tissue samples (5% polyvinylpyrrolidone (mw 40,000) was added to the extraction buffer). The DNA was further purified using the BioRad Prep-A-Gene™ DNA Purification Kit. The RAPD protocol was a modification of the Williams et al. (1990) procedure (C. D. Nelson, pers. comm.). RAPD reactions were performed in 25 μ l volume of 10x buffer (10 mM Tris-Cl, pH 8.3; 50 mM KCl), 2.0 mM of $MgCl_2$, 100 μ M each of dATP, dCTP, dGTP, and dTTP, 0.2 μ M of primer, 2.5 ng of template DNA, and 1.0 unit of *Taq* DNA polymerase and overlaid with 50 μ l of mineral oil. A 96-well MJ Research Programmable Thermal Controller® set for 41 cycles of 1 min at 92 °C (denaturation), 1 min at 35 °C (primer annealing), and 2 min at 72 °C (primer extension) was used for amplifying the DNA. The amplified products were separated in a 1.4% agarose gel in 1x TAE buffer.

Based on the screening of 56 Operon primers (C1-20, F1-20, W1-16) on bulked samples of pecan DNA, 9 primers were selected to fingerprint the individuals in the provenance test and to verify the inheritance of the RAPD markers in the three pedigrees. In addition, 25% of the total number of reactions from the provenance test and 100% of the total reactions for the inheritance study were repeated in order to determine if the DNA banding patterns were reproducible. Negative controls, in which no template DNAs were added to the reaction mixtures, were run for each primer.

Polymorphic and monomorphic loci were scored as either present or absent. Any amplified products found in the pecan sample lanes that corresponded to products in the negative control lanes were not included in the data set. Also, any loci that were not scorable due to clarity for all the individuals in the provenance test were not included. Chi-square analysis was conducted on the loci of individuals from the Brownwood orchard to determine if the loci were inherited in a Mendelian fashion.

The provenance data set was divided into three subsets based on the heritability and the reproducibility of the DNA banding patterns. The first included only the loci that were inherited in a Mendelian fashion and were reproducible in both the inheritance study and in the provenance test. The second subset included all of the heritable loci, whether they were reproducible or not. The third subset consisted of all of the reproducible markers, whether or not they were inherited in a Mendelian fashion.

Cluster analysis of the full data set and the three subsets was performed using Hennig86 vers. 1.5 (Farris 1988), which was distributed to R. M. Zink by the program designer, J. S. Farris. Cladograms of each of the data sets were created using a heuristic search option due to the large number of individuals in the data sets. From the first 100 equally parsimonious trees, a consensus tree was formed for each data set and was used for analysis.

RESULTS

Out of the 56 primers screened, 10 produced no amplification or poor amplification, 20 amplified only monomorphic loci, and 26 amplified at least 1 polymorphic locus. From the 26 primers that produced polymorphic bands, 9 primers were selected to fingerprint the individuals from the Brownwood orchard and the provenance studies (C6, C12, F7, F14, W3, W4, W5, W8, and W16).

From the individuals in the inheritance study, a total of 58 loci, ranging from 0.4 kb. to 2.5 kb., were produced with the 9 primers. After repeating all of the RAPD reactions, 44 of the 58 markers were reproducible. Chi-square analysis was conducted on the presence:absence ratios of the individuals to determine if they were inherited in a Mendelian fashion. None of the observed ratios was rejected ($\alpha = 0.05$), with the exception of cases where a marker was scored in some or all of the progeny but not in either of the parents, which accounted for approximately 25% of the markers. Of the 58 loci, 44 were inherited in a Mendelian fashion, and 38 were heritable and reproducible.

From the individuals in the provenance test, a total of 63 loci, 47 polymorphic and 16 monomorphic, ranging from 0.4 kb. to 2.5 kb., were scored based on the 9 primers chosen. Of the 63 loci, 42 were reproducible based on a check of 25% of the total number of RAPD reactions, and 54 were also scored in the individuals from the inheritance study. Based on chi-square analysis of the presence:absence ratios of the loci scored in the inheritance study, 30 loci were found heritable and reproducible. Only 28 of these loci (16 polymorphic loci) were heritable and reproducible in both the inheritance and provenance studies, and 13 loci were found heritable and nonreproducible in at least one of the studies.

Due to the problems encountered with the reproducibility of the markers, we were interested in how reproducibility and heritability of the markers would affect the clustering of the data when analyzed by Hennig86. Therefore, the full data set of 63 markers (47 polymorphic) for the 77 individuals was divided into three subsets as described previously. The cladograms for the full data set and the heritable and reproducible data subset are shown in figures 1 & 2 respectively. The full data set produced a 356-step consensus tree with a consistency index (CI)=0.13 and a retention index (RI)=0.57. The heritable and reproducible data set produced a 41-step consensus tree with a CI=0.39 and RI=0.81. Both CI and RI can range from 0.0 to 1.0, with values closer to 1.0 indicating a close fit between the data and the tree. The full data set revealed more clustering than the heritable and reproducible data set, but none of the cladograms showed strong congruence to anticipated patterns based on geography.

DISCUSSION

The results of the Hennig86 analysis for all four data sets show only limited clustering of the individuals by geographic location, with the exception of a group of individuals from Louisiana and Mississippi. With the addition of the less reliable data (nonreproducible, nonheritable, or both), more clustering of individuals or populations was apparent, but the CI and RI values decreased. Not all of the clusters included samples from the same or nearby populations. In fact, in most cases, all of the individuals from the same population did not group together, which would indicate that there is a significant degree of variation within populations as well as among them.

The nature of the RAPD markers may also introduce sources of error into the phylogenies. One of the limitations of RAPDs is that it is not known what level of variation in the genome is being detected by the RAPD markers. For example, it cannot be easily determined if a RAPD marker represents the presence of a single copy gene or a multicopy gene. The absence of a marker representing a multicopy gene will only occur if all of the loci are not present. However, the number of present loci of a multicopy gene may be phylogenetically important. In this situation, using RAPD analysis may result in the underestimation of the actual amount and distribution of variation of RAPD markers.

In order to have the highest level of confidence in the information obtained from RAPD analysis, a complete genetic system of the study species, including several generations of parents, progeny, and backcross individuals, should be available. RAPD primers can then be screened on the individuals in the system and further analysis utilize markers that segregate in a Mendelian fashion. The limited availability of pedigrees to evaluate the RAPD markers limits

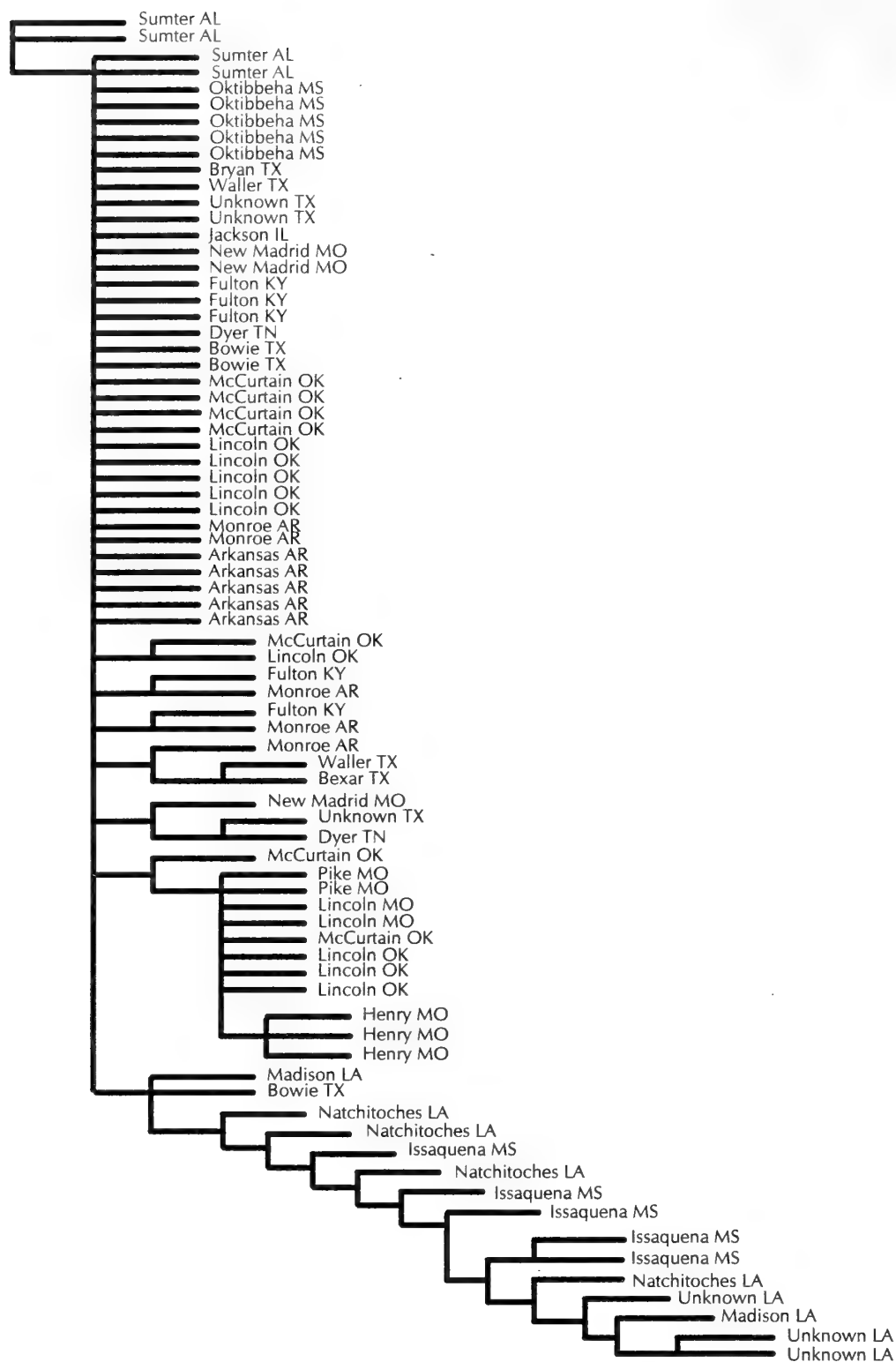


Figure 1. Cladogram generated by Hennig86 using all of the identified marker.

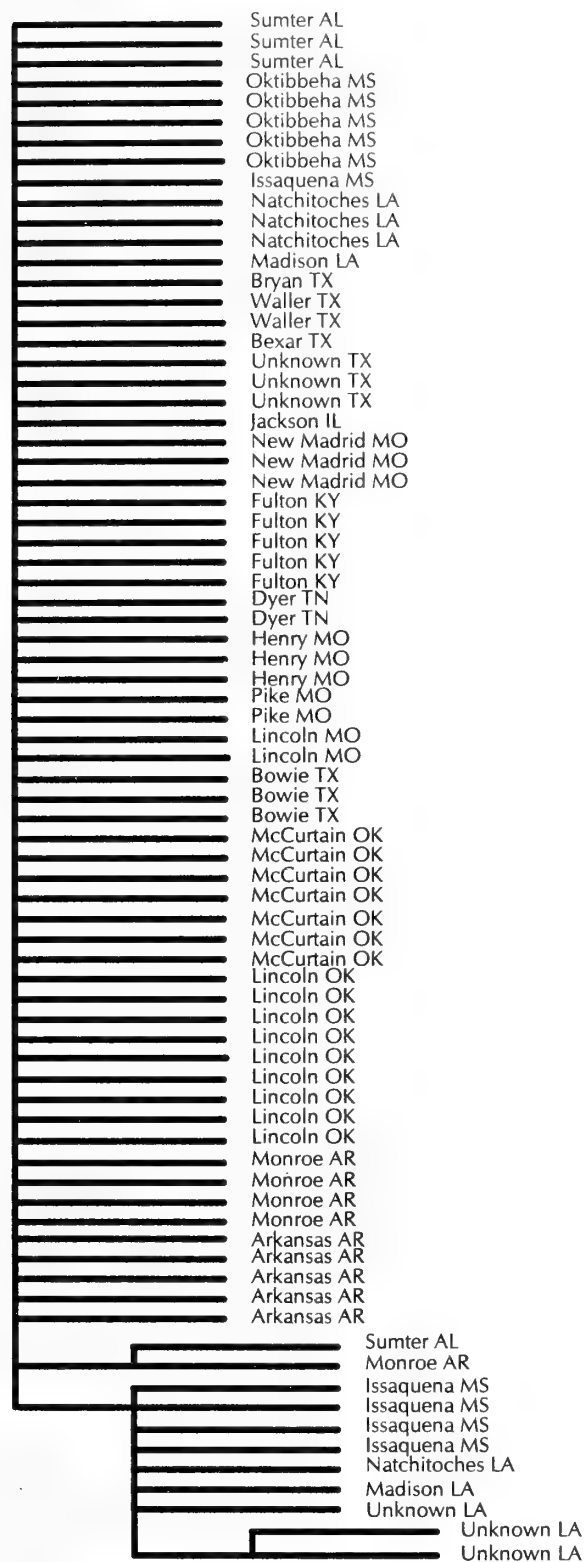


Figure 2. Cladogram generated by Hennig86 using only the heritable and reproducible markers.

the applicability of this technique in phylogenetic analysis and many studies involving wild populations. This may not be a major obstacle for plant species that have an annual life cycle; but for tree species, the long generation times and absence of large pedigrees for most species is a considerable problem.

For this study, only three pedigreed families were available to screen primers and select markers demonstrating Mendelian inheritance. Because of the small sample size, some markers that were not inherited in a Mendelian fashion may have been included in the data set. A larger number of progeny and having more than one generation would reduce this problem. Additionally, none of the three pedigreed lines were from individuals in the provenance test. While it may be acceptable to assume that the markers found in the inheritance study were comparable to those identified in the provenance test, having the genetic system for the individuals in the provenance test would increase the reliability of the results.

The problems of nonreproducible and nonheritable data are not the only possible reasons for the lack of clustering according to geographic location. The natural history of pecan provides additional theories for the degree of randomness demonstrated in the clustering of individuals. It is believed that pecan originated 70-135 million years ago during the Cretaceous period (Stuckey and Kyle 1925), with the present range of pecan determined since the last glaciations of the Quaternary period (Delcourt and Delcourt 1987). Pecan spread northward from the southern part of its present range primarily through the activities of humans (Bettis et al. 1990). Native Americans were primarily responsible for further expansion of the range of pecan during the 17th and 18th centuries (Hume 1906, Stuckey and Kyle 1925, Woodruff 1979). By the mid-1700s, fur traders had introduced the pecan nut to English settlers in the eastern United States. By collecting pecans in one area and planting them in another, they succeeded in extending the range of pecan and in scattering genotypes into regions where they would not have normally been introduced. The spread of the natural range of pecan by humans could help explain the overall lack of meaningful clustering of individuals in the cladograms.

In conclusion, the development of the RAPD technique may provide a new DNA-based marker that can be obtained through relatively simple and inexpensive means. However, the reliability of these RAPD markers must be improved in order to obtain a higher level of confidence in some of the studies using the technique, particularly those involving phylogenetic analyses and wild populations. Modifying the protocol to increase the reproducibility of the markers and evaluating the inheritance of the markers through a complete genetic system consisting of several generations of parents and progeny are two possible methods of increasing the reliability of the RAPD technique.

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SOMATIC EMBRYOGENESIS AND GENE TRANSFER IN AMERICAN CHESTNUT

D. T. CARRAWAY, H. D. WILDE, AND S. A. MERKLE¹

Abstract. -- Leaves, stem sections, ovules and immature zygotic embryos of American chestnut were used as explants in experiments designed to induce formation of somatic embryos. Embryogenic cultures were induced from immature zygotic embryos under the influence of 2,4-D and BA and could be maintained on either semisolid medium or as suspensions in liquid medium. Suspension cultured cells were bombarded using gold particles coated with plasmid DNA encoding β -glucuronidase (GUS), and neomycin phosphotransferase (NPT II). Stable integration of the introduced GUS gene in proliferating American chestnut cells was confirmed via fluorescence analysis, histochemical analysis, and Southern hybridization.

Key words: Castanea dentata, Cryphonectria parasitica, microprojectile bombardment, chestnut blight.

INTRODUCTION

American chestnut, Castanea dentata (Marshall) Borkhausen, was once a major component of climax forests in the eastern United States (Anagnostakis 1987). Until the early 1900s, as much as 25% of some eastern hardwood forests were composed of American chestnut (Burnham 1988). This species was the premier hardwood of the eastern United States by virtue of its abundance, decay resistant wood, rapid growth, size, ability for natural regeneration, and its myriad uses (Clapper and Gravatt 1943). During the early 1900s, American chestnut was nearly destroyed by chestnut blight which is caused by a parasitic fungus, Cryphonectria parasitica (Murrill) Barr, = Endothia parasitica (Murrill) P. J. and H. W. Anderson (Newhouse 1990). Chestnut blight is the most destructive forest disease known (Newhouse 1990). According to some estimates, the value represented by American chestnut, as it existed in the early 1900s would have been nearly \$800 billion in 1991 dollars (Stoke 1940). In spite of attempts to control its advance, less than 50 years after its introduction, chestnut blight had reduced a species that once existed as a large, long-lived forest tree to a short-lived, understory shrub which persists only because of its ability to sprout profusely and repeatedly from living stumps and roots (Beattie and Diller 1954).

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American chestnut could potentially be restored as a component of eastern hardwood forests if a solution to the problem of mortality due to the chestnut blight fungus could be found. While some previous efforts to control chestnut blight have produced limited success, none have yet succeeded in restoring American chestnut to its former status. Control methods attempted to date include: clonal propagation and hybridization of native trees thought to possess low levels of resistance (Elkins et al. 1992); production of American chestnut-like hybrids between native trees and resistant Asian chestnuts (Burnham 1988); colchicine induced production of American chestnut polyploids (Dermen and Diller 1962); development of blight resistance in native trees by radiation mutagenesis (Dietz 1978); and use of hypovirulent fungal strains to reduce the degree of blight pathogenicity (Van Alfen et al. 1975).

Molecular biology techniques may have considerable impact on efforts to control chestnut blight. Choi and Nuss (1992) genetically engineered the chestnut blight fungus with a gene that may lead to a greater incidence of hypovirulence in chestnut blight fungus populations. However, hypovirulence alone is unlikely to bring about the restoration of American chestnut as a dominant forest tree species. Although hypovirulent fungal infections are normally nonlethal, American chestnut stems infected with hypovirulent strains are often badly damaged, and multiple infections, even with hypovirulent strains, can be lethal. Thus, there remains a need to develop American chestnut trees which are resistant to infection by the chestnut blight fungus. We believe that American chestnut trees which have been genetically engineered to be resistant to chestnut blight will, in combination with hypovirulent fungal strains, provide an integrated system of disease control that would be superior to either one alone.

Similar to the progress noted above with the chestnut blight fungus, recent advances in the fields of plant tissue culture and plant genetic engineering have provided powerful, new techniques that can be applied to development of an effective control for the chestnut blight fungus from the host side of the interaction. Recently, there has been increasing interest in somatic embryogenesis due to the tremendous potential of its application to plant improvement via high-volume, large-scale clonal propagation of elite genotypes. Successful transformation of embryogenic cells via microprojectile bombardment and subsequent regeneration of transgenic trees has been demonstrated in walnut, Juglans regia L., (McGranahan et al. 1988), yellow-poplar, Liriodendron tulipifera L., (Wilde et al. 1992), and white spruce, Picea glauca (Moench) Voss, (Ellis et al. 1993). Use of genetic engineering in conjunction with somatic embryogenesis could make possible the development of a practical, reliable gene transfer system for American chestnut. This gene transfer system may ultimately be used to incorporate fungal resistance genes into the American chestnut genome.

No genes that code for products known to inhibit the growth of chestnut blight fungus *in vivo* have yet been isolated and cloned. Our goal is to develop a reliable system for generating transgenic American chestnut trees, so that when such genes are cloned, there will be a protocol readily available to engineer American chestnut trees with them. Thus, our approach involves achievement of two objectives: (1) To develop a system for *in*

vitro propagation of American chestnut via somatic embryogenesis, and (2) To define a protocol for incorporation and expression of foreign DNA in embryogenic American chestnut cells. Methods developed to achieve each separate goal will then be combined in order to produce transgenic trees from the transformed cell cultures via somatic embryogenesis.

MATERIALS AND METHODS

Explant Material

American chestnut ovules and zygotic embryos from developing burs were used as explants to initiate cultures. Explant material was collected from as many locations and genotypes as possible throughout the original American chestnut range. Since fertile American chestnut trees are not common, locations and available genotypes were dictated by the occurrence of fertile trees. During the two years (1991 and 1992) that cultures were initiated, twenty-five trees from locations in New York, Wisconsin, Connecticut, Pennsylvania, North Carolina and Georgia were sampled. Fruit from 5 different maturity stages was sampled in an attempt to include material within the developmental "window" required for the development of embryogenic cultures.

Culture initiation

Out-of-state collections of American chestnut material were sealed in plastic bags and shipped via overnight mail. In-state material was sealed in plastic bags and transported in an ice chest. All plant material was refrigerated upon arrival. Nuts were surface sterilized according to a procedure previously described for American chestnut (Merkle et al. 1991). Ovules were wounded before being placed onto culture medium. Cotyledons were removed from the embryonic axis of immature zygotic embryos and were cultured separately. Mature zygotic embryos were cultured in the same fashion except that cotyledons were cut into four quadrants before being placed onto culture medium.

Culture Media and Growth Regulators

Explants were cultured on two types of semisolid basal medium, woody plant medium [WPM] (Lloyd and McCown 1980) and Driver and Kuniyuki medium [DKM] (McGranahan et al. 1988). Media were solidified with 0.8% (wt/v) phytagar. During the two years that cultures have been initiated, four auxins and two cytokinins have been tested. Auxins tested were 2,4-dichlorophenoxyacetic acid (2,4-D), indole-3-acetic acid (IAA), naphthaleneacetic acid (NAA), and 4-amino-3,5,6-trichloropicolinic acid (picloram). Cytokinins tested were benzyladenine (BA) and thidiazuron. Plant growth regulator treatments were as follows: (A) no growth regulators; (B) auxin only; (C) cytokinin only; (D) auxin + cytokinin (E) 1, 2, or 3 week pulse on B followed by transfer to A or C; (F) 1, 2, or 3 week pulse on C followed by transfer to A or B; (G) 1, 2, or 3 week pulse on D followed by transfer to A.

Microprojectile Bombardment

Suspension cultures were initiated by inoculating cells from cultures on semisolid medium into 125 ml Erlenmeyer flasks containing liquid WPM supplemented with 3 mg/l 2,4-D and 0.25 mg/l BA (W3.25). Ten day old suspension cultures were fractionated through a stainless steel mesh with 380 μ m grid size. The fraction that passed through the mesh was plated on 90 mm sterile filter paper disks over semisolid W3.25. After ten days growth on filter paper disks, the cells were bombarded with plasmid DNA (pBI121.1 [Jefferson et al. 1987]) which carries the *uidA* reporter gene encoding β -glucuronidase (GUS), and the *nptII* selectable marker gene encoding neomycin phosphotransferase. Plasmid DNA was precipitated onto 1 μ m diameter gold particles using the CaCl_2 method of Klein *et al.* (1989). Ten microliters of an alcohol suspension of the DNA-coated gold particles were dried onto a macrocarrier and accelerated into target cells using a BioRad PDS-1000/HE particle gun.

Selection of transgenic cells

Prior to microprojectile bombardment, a kanamycin sensitivity curve was developed for 3 different lines of embryogenic American chestnut cells. Cell lines were subcultured to W3.25 supplemented with kanamycin sulfate in concentrations ranging from 20 to 200 μ g/ml in 20 μ g/ml increments. After 2 weeks on the selection medium, all cells that had been subcultured to medium containing more than 80 μ g/ml kanamycin sulfate had been killed. Bombarded cells were allowed to stabilize for 12 days on antibiotic free medium before being transferred to selection medium with 100 μ g/ml kanamycin. After 8 weeks on selection medium, kanamycin-resistant cell clusters were transferred to fresh selection medium and maintained by monthly subcultures to fresh selection medium.

GUS Assays

Expression of the *uidA* gene in kanamycin resistant cells was verified with a histochemical assay using 5-bromo-4-chloro-3-indoyl glucuronic acid (X-gluc). Approximately 10 mg of kanamycin resistant cells per well were placed in 96-well tissue culture plates with 20 μ l of 2mM X-gluc solution per well. A fluorescence assay was also performed using 4-methyl umbelliferyl glucuronic acid (MUG). A protein extract was prepared by homogenizing 100 mg of cells in 1.5 ml Eppendorf tubes containing 200 μ l of GUS extraction buffer (Jefferson et al. 1987). The homogenate was centrifuged at 15 g for 1 minute and the supernatant was removed and split into two equal parts. An equal volume of GUS extraction buffer containing 2 mM MUG was added to the protein extracts. The reaction in one tube was stopped immediately with 100 μ l of 200 mM Na_2CO_3 . The other half of the protein extract/MUG reaction was incubated at 37° C for one hour and stopped. Fluorescence, as visualized with long wave UV light, was compared between the two reactions.

DNA isolation and analysis

A cetyltrimethylammonium bromide nucleic acid extraction procedure modified from Doyle and Doyle (1990) was used to isolate DNA from approximately 150 mg of callus tissue from seven different GUS positive cultures and one nontransformed control. Isolated DNA was digested with the restriction enzymes EcoRI and HindIII. Digested DNA from the seven putative transformants (10 µg/well), and the EcoRI/HindIII excised GUS fragment from pBI121.1 were separated by electrophoresis through a 1% agarose gel. Southern blot hybridizations (Southern 1975) were performed on the resulting gel. The probe was a randomly labeled EcoRI/HindIII excised GUS sequence from pBI121.1. Probe labeling, hybridization, and detection was performed as per the protocol in the Boehringer Mannheim Genius III digoxigenin DNA labeling and detection kit.

RESULTS AND DISCUSSION

Somatic Embryogenesis

Embryogenic response varied according to tree source, type and maturity stage of explant tissue, and growth regulator treatment. Explants from 16 of the 25 trees sampled during 1991 and 1992 produced somatic embryos. However, only 5 trees produced cell lines that exhibited repetitive somatic embryo production over several months. Repetitive embryogenic cultures were initiated from developing ovules and zygotic embryos less than 4 mm in length (Figure 1). The cell lines that consistently produced somatic embryos had been continuously exposed to a combination of 3 mg/l 2,4-D and .25 mg/l BA. There was no noticeable difference in culture performance between the two basal media.

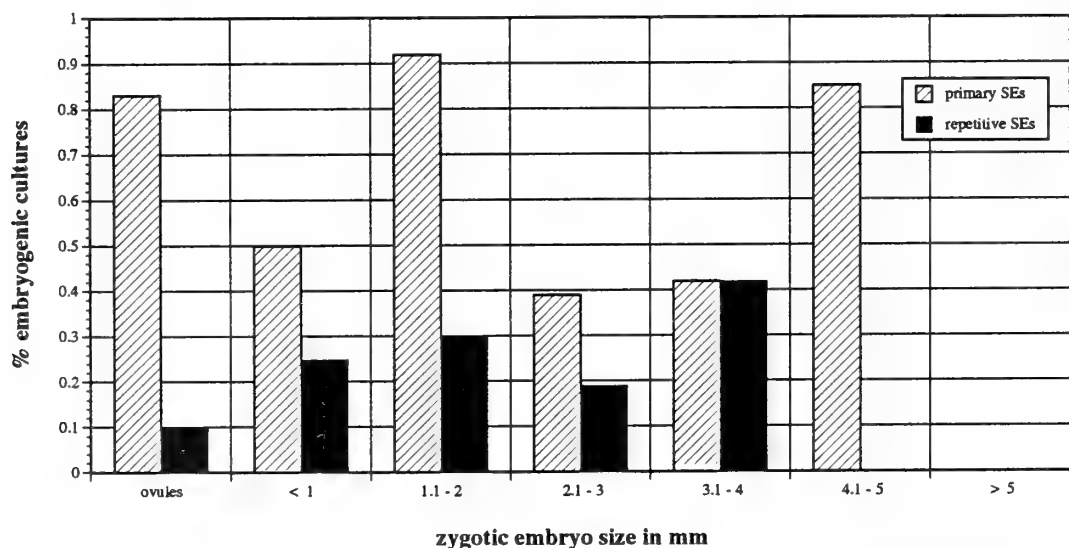


Figure 1. Relationship of zygotic embryo maturity stage (measured by length in mm) and embryogenic frequency after 3 months on W3.25 medium.

Callus from two cotyledonary explants produced adventitious shoots. Non-expanded leaves and primary growth of stem sections from these adventitious shoots were explanted to the same medium used in production of embryogenic cultures from immature zygotic embryos. Nonembryogenic callus resulted from the vegetative explants.

Embryogenic cultures are being maintained on semisolid by monthly transfer to W3.25. Somatic embryos can be produced from these cell lines after subculture to WPM without growth regulators, but with the addition of 1% wt/v activated charcoal. American chestnut somatic embryos are now receiving treatments in order to promote maturation and germination. These treatments include addition of a non-plasmolyzing osmoticum, altering the carbon source, cold stratification, desiccation, and exposure to abscisic acid.

Gene Transfer

Microprojectile bombardment produced 16 putatively transformed cell lines. Approximately 8 weeks after bombardment, kanamycin-resistant colonies of cells were visible against a background of senescent cells. Each resistant colony was transferred to its own plate of fresh selection medium to continue growth. Approximately 10 weeks later, each putatively transformed line was tested for expression of the GUS reporter gene, using both the fluorescence assay and the histochemical assay. Of the 16 kanamycin-resistant lines, 11 were GUS positive and 5 had no GUS activity detectable by the X-gluc or MUG assays. Following 6 months of maintenance on selection medium, Southern blot analysis was performed on seven GUS positive cell lines. The GUS gene was detected in all seven lines (Fig. 2). To date, no mature somatic embryos have been produced from these transformed cell lines. However, all of the transformed lines were derived from a single embryogenic American chestnut suspension culture which had only infrequently produced well-formed somatic embryos prior to the gene transfer experiments. Other, more highly embryogenic lines are available for bombardment, and we intend to apply what we have learned to date to obtain transgenic somatic embryos from those lines.

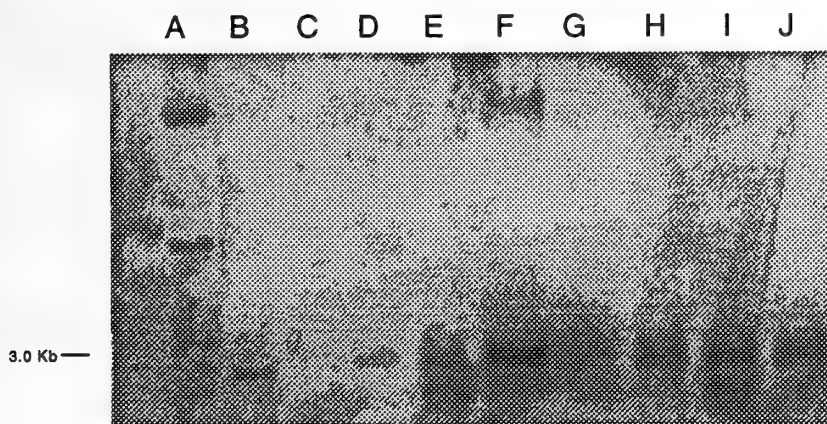


Figure 2. Southern blot analysis of the GUS gene in transformed American chestnut cultures. Lane A, DNA molecular weight markers. Lane B, pBI121.1 digested with EcoRI and HindIII. Lane C, nontransformed cell line. Lanes D-J, transformed cell lines.

CONCLUSION

We have demonstrated that embryogenic cultures can be induced from immature zygotic embryos of American chestnut. In addition, we have shown that microprojectile bombardment is an effective method for genetic engineering of American chestnut cells. We believe that the integration of somatic embryogenesis and microprojectile-mediated gene transfer will allow development of a system for producing transgenic American chestnut trees.

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TRANSFORMATION OF SLASH PINE

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Abstract.--Genes responding to environmental stresses are being identified in Pinus and other woody plant species. The development of Pinus tissue culture systems are needed in order to: (1) characterize stress responsive genes in Pinus, and (2) to transform Pinus species with stress responsive characteristics. An embryogenic tissue culture system has been developed for slash pine (Pinus elliottii Engelm.) with the objective of understanding gene function as well as for developing technology for gene transfer and genetic transformation. Plantlets have been produced via embryogenesis. Foreign gene transient expression has been obtained in cell suspensions using a gas-driven particle bombardment procedure. A more stable, transformed callus has been obtained after bombardment with a gene providing resistance to the antibiotic, kanamycin. This progress in the development of transformation technology will help us understand the role(s) of stress-responsive genes in Pinus.

Keywords: Pinus elliottii Engelm., transformation, in vitro culture, embryogenesis, particle bombardment.

INTRODUCTION

Slash pine (Pinus elliottii Engelm.) is one of the hard yellow pines indigenous to the southeastern United States (Lohrey and Kossuth 1990). It is one of the two southern pines used for naval stores and is one of the most frequently planted timber species in North America (Lohrey and Kossuth 1990). It is favored by many forest managers because of its fast growth and excellent utility for pulp, lumber, and poles (Sheffield et al. 1983). It has the smallest native range of the four southern pines and grows naturally from South Carolina south to central Florida and west to Louisiana. It has been established by planting as far north as Tennessee and as far west as eastern Texas where it now seeds naturally (Lohrey and Kossuth 1990). In the 3 decades prior to 1980, the slash pine ecosystem increased by 22% and peaked in the late 1970s and early 1980s (Sheffield et al. 1983). Today, the rate of planting outside the natural range has slowed compared to the 1950 to 1970 period.

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Slash pine seed source planting trials outside the natural range indicate that it is not well adapted to extreme northern and western environment stresses such as low temperature and extreme drought (Switzer 1959; Snyder et al. 1967). Furthermore, slash pine is most susceptible to the fungal fusiform rust disease (Blakeslee 1983). Many trees are killed due to these stresses, and because of this there is a much lower amount of slash pine acreage planted each year compared to other Pinus species. For example, out of 27,000 acres of Pinus plantings administered by the Texas Forest Service in 1992, less than 100 acres were planted with slash pine (Barber 1993).

Much research has been conducted relative to identifying genetic variation in conifers in response to disease (Wright 1976; Zobel and Talbert 1984) and drought (Van Buijtenen et al. 1976; Pallardy 1981; Larsen 1981; Newton et al. 1992). Furthermore, many of these responses can be characterized at the molecular and gene level (Newton et al. 1991; Tauer et al. 1992; Funkhouser et al. 1993), and genetic transformation has been suggested as one of the alternatives for providing tolerance to both disease (Lamb et al. 1992) and drought (Newton et al. 1991; Funkhouser et al. 1993). For example, genes encoding a chitinase or a ribosome-inactivating protein (Lamb et al. 1992) could be tested for their conference of protection against fusiform fungal attack. In our own laboratories, we have identified a methyltransferase gene from Pinus taeda (J. Cairney, S. Chang, R. Newton, unpublished) which is homologous to a parsley gene (Schmitt et al. 1991), and whose over-expression could modify lignin deposition (Sederoff et al. 1991). Lignin deposition is one of several multigenic defenses known for protection against microbial pathogens and it is proposed as a strategy for manipulation (Lamb et al. 1992).

With new biotechnologies being developed for plant transformation, it appears that they could be used on behalf of slash pine, i.e. to modify the species' tolerance to either drought and/or disease stress. Two technologies that are rapidly advancing in development for this purpose are: (1) gene transfer, and (2) in vitro culture. Recent advances in genetic transformation via the biolistic process represent a new approach to the problem of how to deliver DNA into intact conifer cells. Use of microprojectile-mediated DNA transfer has been reported for embryogenic cells of Picea glauca (Ellis et al. 1991; 1993), P. abies (Newton et al. 1992; Yibrah and Clapham 1990), P. mariana (Duchesne and Charest 1991), and the cotyledons of Pinus taeda (Stomp et al. 1990). In addition to DNA delivery capabilities, in vitro culture technologies with conifers are also advancing, particularly those associated with embryogenesis (Tautorus et al. 1991; Gupta et al. 1993).

Embryogenesis is the process of embryo formation from the zygote. The term somatic (or asexual) embryogenesis is applied when the embryo originates from cells that are not the product of gametic fusion. Somatic embryogenesis in conifers has progressed since the first report in 1985 (Hakman et al. 1985). Currently, the development of somatic embryos (SEs) on solid media has been reported in Norway spruce (Picea abies (L.) Karst) (Hakman et al. 1985; Krogstrup 1986; von Arnold and Hakman 1986) sugar pine (Pinus lambertiana Dougl.) (Gupta and Durzan 1986), slash pine (Pinus elliottii Engelm.) (Jain et al. 1989), loblolly pine (Pinus taeda L.) (Becwar et al. 1990; Gupta and Durzan 1987), white spruce (Picea glauca (Moench) Voss) (Hakman and Fowke 1987), and black spruce (Picea mariana (Mill.) B.S.P.) (Hakman and Fowke 1987). Spruces, larches, firs, and pines have been the focus of many studies,

and many have yielded successful results although plantlet recovery is often poor (Hakman and von Arnold 1985; Atree et al. 1990; Tremblay 1990).

Research in our laboratories has focused on the following three objectives: (1) identifying and characterizing stress-induced genes in Pinus, (2) developing suitable in vitro regenerations systems in Pinus, and (3) developing suitable DNA transfer protocols for Pinus transformation. In this paper, we report a successful regeneration system for Pinus elliottii, and our progress in regard to tissue transformation in this species.

MATERIALS AND METHODS

Plant materials

Weekly collections of green seed cones from one slash pine orchard tree (S2PC1) were made from an open-pollinated orchard of the Texas Forest Service in Magnolia Springs, Texas during the time interval of June 11, 1991 to July 2, 1991. The cones were prepared as described by Marek-Swize et al. (1994).

Initiation and maintenance of embryogenic callus

Immature zygotic embryos were aseptically excised from the seeds under a dissecting microscope. To initiate calli, explants were plated on two variations of a Douglas Fir Cotyledon Revised media (DCR) (Gupta and Durzan 1986) solidified with 1% gelrite as described by Marek-Swize et al. (1994). After 4-6 weeks when induction had been accomplished, all embryogenic calli were placed on a modified DCR-F media (Finer et al. 1989) with growth regulator concentrations reduced to one-fifth proportions (2,4-D, 1.8 μ M; BA, 0.9 μ M) and sucrose levels elevated to 30g/L (DCR-F/5). Explant evaluations were made biweekly for their potential to form embryogenic calli, and they were subcultured biweekly. After several passages, the DCR-F hormone levels were reduced to one-tenth of their original levels (DCR-F/10) for long term maintenance and proliferation.

Somatic embryo (SE) maturation

Because prior protocols associated with slash pine SE maturation (Jain et al. 1989) were not successful, the established embryogenic calli were subjected to the following subculture protocol: embryogenic calli with Stage 1 SEs were transferred from DCR-F/10 medium to DCR medium containing activated charcoal (1%, w/v) and sucrose (30 g/L) for 1 week, followed by subculture on DCR medium containing sucrose (30 g/L), ABA (10 μ M), and BA (1 μ M) for 4-5 weeks, and a final subculture on DCR medium containing sucrose (30 g/L).

For maturation to plantlets, Stage 3 SEs were immersed upside down in a DCR-agar medium and culture plates were inverted according to procedures of Becwar et al. (1989) at 25°C and light intensity of 250 μ molm⁻² sec⁻¹ until roots developed. The rooted plantlets were transferred to tubes containing a DCR medium with sucrose (30g/L) for root elongation until they reached a size of 2-3 cm. They were transferred to 8 cm pots containing peat, perlite and vermiculite (1:1:2) and allowed to acclimate in plastic bags for 4 weeks before transferring to the greenhouse.

Cell Suspensions

Embryogenic calli were initiated from immature zygotic embryos (Marek et al., 1994), and subcultured every 2 weeks in darkness at 25°C. Embryogenic cell suspensions were established from embryogenic calli pieces introduced into liquid DCR medium. They were cultured in 250 ml flasks containing 50 ml of medium in darkness at 25°C on a rotary shaker at 150 rpm. They were subcultured every 2 weeks. Two hundred mg of tissue were spread evenly on a filter disk in the form of a thin, circular layer (diam=5.5 cm) using a Buchner funnel with vacuum. The disks were placed on the solid medium before and after bombardment. In some cases the solid medium contained ABA (10µM).

Plasmids

The Dc8/GUS plasmid was obtained from Z. R. Sung, Univ. of Calif., Berkeley, and contains the GUS structural gene fused between the ABA-responsive, 1.5 kb 5' upstream region of the Dc8 gene isolated from carrot (Hatzopoulos et al. 1990) and the nopaline synthase polyadenylation sequence, cloned into a pUC plasmid, total size 6.6 kb. The Dc3/GUS plasmid was obtained from T. L. Thomas, Texas A&M Univ., and contains the GUS structural gene fused to a 1.5-kb 5' upstream element of the carrot promoter, Dc3 (Seffens et al. 1990). The 70S/GUS plasmid (pJIT65) contains a GUS structural gene, a double tandem 35S promoter and a 35S polyadenylation sequence (Guerineau et al. 1990) cloned into a pUC plasmid, total size 5.6 kb. The Em/GUS plasmid (pBM113Kp) was obtained from R. S. Quatrano, Univ. N. C., and contains the ABA-inducible promoter from the Em gene of wheat (Litts et al. 1987) which is linked in translation fusion to GUS and a 3' flanking region from CaMV 35S cloned into the pUC derived plasmid (pBM113Kp). For kanamycin resistance and stable transformation, the plasmid pB101.5 constructed in our laboratory (S. Chang and N. Dong) containing nos/NPTII and Dc8/GUS, was used.

Preparation of Particles

DNA was precipitated onto gold particles using the CaCl₂ precipitation procedure of Klein et al. (1988); 10 µg of supercoiled plasmid DNA added to 3 mg gold particles [diam = 1.6 µm (BioRad)] were finally suspended in 50 µl ethanol. Aliquots of 10 µl were pipetted onto the macrocarrier.

Bombardment

The Dupont BiolisticTM particle delivery system (PDS-1000) was modified with a helium gas driven system. Each filter disk was bombarded while on the medium. To optimize bombardment conditions, the filter disks were placed at a distance of 74 mm from the stopping screen, under a chamber vacuum of 28.5 inch Hg, and bombarded with a rupture disc of 650 psi. After bombardment, the filter paper disk on the solid medium was incubated at 25°C for 2 d.

GUS Assay

β-glucuronidase expression was histochemically assayed with 5-bromo-4-chloro-3-indolyl glucuronide (X-GLUC) (Jefferson 1987) 2 d after bombardment.

Five hundred μ l of X-GLUC were applied to tissues on each disk contained in petri dishes and maintained at 37°C in darkness for another 1 d. Blue spots were counted under a dissecting microscope and the mean number and standard error were computed for each treatment. Spot number is correlated with the fluorimetric enzyme activity assay (Ellis et al. 1991).

Callus Growth Measurement

With stable transformation studies, callus growth was quantified by measuring their diameters from photographs taken at the beginning of the experiment and 20 days later.

RESULTS AND DISCUSSION

Slash Pine Embryogenesis

The time of cone collection spanned over a 4 week period. These explants produced embryogenic calli at an overall rate of 18% (Table 1). Within 4-6 weeks on induction media, callus development had begun on the explants. Calli appeared to develop from explant suspensor cells at the base of the developing embryo head. Calli derived from the explants proliferated rapidly and maintained their embryogenic potential with Stage 1 SEs. For somatic embryo (SE) development and maturation, embryogenic calli with Stage 1 SEs were subcultured. This subculturing protocol produced not only a high number of Stage 3 SEs, but also sixty-five Stage 4 plantlets (Table 1). Presently, nineteen plantlets have been placed in pots.

Table 1. Frequency of embryogenic calli and number of Stage 3 somatic embryos and Stage 4 plantlets of Pinus elliottii.

<u>Frequency of embryogenic calli/explant</u>	<u>Number of Stage 3 Somatic Embryos</u>	<u>Number of Stage 4 Plantlets</u>
85/454	215	65

In 1988, researchers in our laboratory were the first to establish embryogenic calli from immature zygotic embryos of slash pine (Jain et al. 1989), and we reported for the first time the maturation of slash pine embryogenic calli into plantlets (Marek-Swize et al. 1994). Along with loblolly pine (Pinus taeda) (Becwar et al. 1990; Gupta and Durzan 1987), this is the second commercially important Pinus species in the Southeastern United States for which this has been accomplished.

Immature zygotic embryos have served as an excellent explant source for SE production in a variety of conifer species. The optimum stage of immature zygotic embryo development as an explant source for initiation of embryogenic tissue in Picea species is postcotyledonary (Hakman et al. 1985; Hakman and Fowke 1987) while that for Pinus species (Finer et al. 1989; Gupta and Durzan 1987; Jain et al. 1989) including slash pine is precotyledonary. Immature

(Hakman et al. 1985; von Arnold and Eriksson 1981) and mature (von Arnold 1987; von Arnold and Hakman 1986; Gupta and Durzan 1986) zygotic embryos appear to be the best explants for initiating embryogenic calli since they have greater degrees of competency than other tissue types. Conifer embryo-genic calli SEs can mature and develop further when placed on auxin-free solid or liquid media (Gupta et al. 1993). In Norway spruce, auxin appears to be required for differentiation of new SEs, and cytokinin is required for retaining somatic embryo organization (Bellarosa et al. 1992).

Abscisic acid also has been shown to enhance SE maturation (Gupta et al. 1993; Durzan and Gupta 1987; von Arnold and Hakman 1988). In addition to an auxin-free ABA-containing medium, enhanced SE maturation also results from an increased osmolality, particularly in early stages of embryo development (Gupta et al. 1993). Osmotica such as sorbitol, mannitol and myo-inositol in the media reduce its osmotic potential and subject SEs to water stress. However, the elevated levels of sucrose that were maintained in all subculture media in our study, may have had an osmotic function in enhancing maturation. With sucrose concentrations ranging from 10 to 50 g/L (30 g/L being optimal), Norway spruce SE maturation was enhanced (von Arnold and Hakman 1986; Hakman and von Arnold 1988).

Slash Pine Transformation

Microprojectile DNA delivery has been demonstrated to be a suitable method for conifer species (Newton et al. 1992; Charest et al. 1993; Stomp et al. 1991). With this technology, we wanted to optimize the delivery system and to assess several promoter sequences as to their capability for driving GUS gene expression in transformed cell suspensions of slash pine. Five promoter sequences fused to the GUS gene were assessed by counting the number of blue spots/disk 48 hr after bombarding with DNA (Table 2). For comparison, the CaMV 35S promoter is often used as a standard. Its performance was compared with the tandem repeat of 35S (CaMV 70S) and the abscisic acid (ABA)-regulated promoters (Dc3, Dc8, and Em). The latter three were selected because: (1) many gene responses to stress are mediated by ABA (Skriver and Mundy 1990; Newton et al. 1991), and (2) somatic embryo maturation in slash pine (Marek-Swize et al. 1994) and other conifers (Gupta et al. 1993) is mediated by ABA. The promoter, Dc8, was also chosen for study because it is not inducible in nonembryonic cells by ABA (Hatzopoulous et al. 1990).

The tandem repeat, CaMV 70S promoter, appeared to be as effective as CaMV 35S in driving GUS expression in slash pine cells suspensions, while the Dc8 promoter showed the least expression (Table 2). A maximum number of 1845 spots/disk were obtained with the Dc3 promoter with one bombardment, and a mean of 764 was obtained with 10 bombardments (Table 2). On the average, the Em promoter-driven GUS expression was less than that driven by Dc3, but was similar to that driven by both DC3 and CaMV 70S (Table 2). Although the number of bombardments was limited, the Dc8 promoter was more responsive to the ABA treatment than was Dc3 (Table 3). Dc8-driven GUS expression was also enhanced by ABA in transformed tissues of Norway spruce (Newton et al. 1992).

Table 2. Promoter-driven GUS expression in embryogenic cell suspensions of Pinus elliottii.

<u>Promoter</u>	<u>Number in Sample (n)</u>	<u>Number of Blue Spots/Disk</u> <u>Mean + S.D</u>	<u>Maximum</u>
35S	10	433±264	979
70S	3	642±195	832
Dc3	10	764±479	1845
Dc8	10	176±63	268
Em	3	694±140	852

Table 3. Effect of abscisic acid (ABA) on promoter-driven GUS expression in embryogenic cell suspensions of Pinus elliottii.

<u>Promoter</u>	<u>Number in Sample (n)</u>	<u>Mean (±SD) Number of Blue Spots/Disk</u> <u>Control^a</u>	<u>ABA^b</u>
35S	3, 3	352±66	339±49
Dc3	3, 3	591±102	374±110
Dc8	3, 3	151±23	296±29

^a 2 days on media without ABA

^b 2 days on media with ABA (10 µM)

These data indicate that there is a high level of GUS expression in slash pine embryogenic cells, but the challenge still remained to sustain the expression as well as regenerating plants from those cells which stably incorporated the introduced genes into their genome. Transformed cell selection with an introduced kanamycin-resistance gene (nos/NPTII) which subsequently leads to stable transformation has been accomplished with white spruce (Ellis et al. 1993). Sublethal kanamycin levels in the media was used to select for transformed cells. We have utilized a similar procedure with nos/NPTII and kanamycin resistance introduced into slash pine cell suspensions.

Slash pine cell suspensions were bombarded with nos/NPTII+Dc8/GUS and converted back to callus. The calli were then subjected to two selection regimes: (1) 45 days on kanamycin media with a concentration of 20 µg/ml, and (2) 30 days on kanamycin with a concentration of 50 µg/ml. Calli derived from non-bombarded suspension cells (Control) and from the bombarded doubly-

selected calli (Bombarded) were then placed on a solid nutrient media containing kanamycin at four different concentrations (20, 40, 80 and 160 $\mu\text{g/ml}$). Callus diameter was measured at the time it was placed on the medium and after 20 days on the same media. The diameter of control calli decreased 0.3 to 0.7 mm on kanamycin media (Table 4). On the other hand, calli derived from bombarded cells increased in diameter on all of the kanamycin media (Table 4). These data indicate that the calli derived from suspension cells bombarded with nos/NPTII have been transformed and are resistant to kanamycin. These calli are still embryogenic and are being subjected to maturation treatments in order to derive transformed somatic embryos.

Table 4. Effect of kanamycin on growth of calli derived from cells with or without bombardment with nos/NPTII+Dc8/GUS and after 20 days on kanamycin media.

Kanamycin Conc. ($\mu\text{g/ml}$)	-----Mean Callus Diameter (mm) ^a -----					
	Control			Bombarded		
	0 days	20 days	Growth	0 days	20 days	Growth
20	2.7	2.3	-0.4	2.2	3.9	+1.7
40	2.7	2.0	-0.7	2.3	3.2	+0.9
80	2.4	2.0	-0.4	1.9	2.5	+0.6
160	2.4	2.1	-0.3	2.2	2.3	+0.1

^a n = 24

With white spruce callus, the ability to form embryogenic callus at a high frequency was superimposed on the ability to suppress, yet not kill the tissue, during selection on kanamycin media (Ellis et al. 1993). The use of sublethal kanamycin levels (5 $\mu\text{g/ml}$) suppressed but did not kill non-transformed cells, while allowing transformed cells to divide (Ellis et al. 1993). The sublethal levels of kanamycin in our study with slash pine appears to be about 20 $\mu\text{g/ml}$ (Table 4), and the transformed calli are still embryogenic. Transformed calli from Norway spruce SEs did not retain embryogenic potential when selected with 10 $\mu\text{g/ml}$ kanamycin (Robertson et al. 1992).

CONCLUSIONS

Embryogenic callus of slash pine was initiated with auxin and cytokinin using immature zygotic embryos as explants. The callus was advanced toward SE maturation resulting in production of plantlets. This is the second commercial species of pine in the southern U. S. in which SEs have been produced. Cell suspensions of these embryogenic calli were placed on filter paper and bombarded with DNA plasmids coated on gold particles. Five different promoters fused to GUS were transiently expressed in slash pine cells, with the Dc3 promoter from carrot providing the greatest GUS driven expression. Calli stably transformed with nos/NPTII resulted from selection on media containing kanamycin. With an available, rapidly growing, conifer culture system, coupled with a suitable DNA delivery system, more progress in biotechnology research can be achieved leading to eventual genetic transformation and understanding gene expression in pines.

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SPECIFICITY OF HOST:PATHOGEN GENETIC INTERACTION FOR FUSIFORM RUST DISEASE ON SLASH PINE¹

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Abstract.-- Multiple stems on individual ramets (rooted cuttings) of 60 slash pine (*Pinus elliotii* Engelm. var. *elliotii*) clones were artificially inoculated using 2 single urediniospore-derived cultures of the fusiform rust fungus, *Cronartium quercuum* (Berk.) Miyabe ex Shirai f. sp. *fusiforme*. Presence or absence of fusiform rust galls were recorded for each inoculated branch at 6 and 9 months post-inoculation. Using disease phenotypes, the cultures were distinguished from each other by several pairs of clones, and the clones could be sorted into distinct groups. Several phenotypic groups appeared to represent specificity between corresponding gene pairs (CGPs) in the host and pathogen, suggesting the presence of a gene-for-gene interaction. Variation in disease phenotypes within clone:cultures combinations was interpreted as segregation at the pathogenicity loci of the CGPs. To utilize this additional information, a modified gene-for-gene analysis was developed and applied to the data. The analysis suggested the presence of four CGPs and clone and culture genotypes were postulated for each CGP. Further inoculation experiments involving these clones and cultures and genetic test crosses of both will be required to verify the hypothetical CGPs and clone and culture genotypes.

Keywords: gene-for-gene interaction, rust resistance, *Pinus elliotii*, *Cronartium quercuum*.

INTRODUCTION

Inoculation studies between members of the *Pinus* subsection *Australes* and various collections of *Cronartium quercuum* have shown host specialization, resulting in the taxonomic distinction of formae speciales within *C. quercuum* (Burdson and Snow 1977). *Cronartium quercuum* f. sp. *echinata* cultures differentially infect *Pinus echinata* Mill. but not *P. taeda* L., *P. elliotii* Engelm. or *P. palustris* Mill., while the opposite holds for cultures of *C. quercuum* f. sp. *fusiforme*. An effort to further characterize resistance and pathogenic variability in *P. e. elliotii* and *C. q. fusiforme* (Griggs and Walkinshaw 1982), resulted in the hypothesis that the interaction may conform to a gene-for-gene system (Kinloch and Walkinshaw 1990). The hypothesis could not be rigorously evaluated because several assumptions about the experimental materials were necessary and experiments to check the assumptions were not conducted.

The literature provides opposing views on the likelihood of a gene-for-gene system between *P. e. elliotii* and *C. q. fusiforme*. Arguments against this system suggest that gene-for-gene specificity is an artefact of plant breeding and thus is very unlikely in a natural system such as the *P. elliotii*:*C. q. fusiforme* pathosystem (Barrett 1985). However Burdon and Jarosz (1988) have shown gene-for-gene specificity in wild populations of *Glycine canescens* and *G.*

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argyrea and the rust pathogen Phakopsora pachyrhizi. Additionally Loegering (1984) argues that much of what is called general resistance is due to specificity and it can be explained by the interaction of corresponding gene pairs (CGPs) in a gene-for-gene system.

Tests of the assumptions made by Kinloch and Walkinshaw (1990) have not been possible due to technical problems in efficiently cloning and breeding both the host and pathogen species. Recent improvements in these technologies have allowed us the opportunity to begin testing the hypothesis of gene-for-gene interaction in the P. e. elliottii:C. q. fusiforme pathosystem. In the present study, we inoculated several clones of slash pine with basidiospore progenies of two single urediniospore derived cultures of the fusiform rust fungus. Because basidiospores represent a segregating population of the single (dikaryon) urediniospore derived culture (Doudrick et al. 1993), basidiospore inoculations present a problem in data analysis under the gene-for-gene model (Flor 1956, 1971; Person 1959; Loegering 1974, 1984). However, given several opportunities to infect each host genotype with each pathogen culture progeny, we reasoned that under certain inoculation conditions we may be able to identify segregating alleles for pathogenicity in each culture. Under these conditions, a more powerful gene-for-gene analysis would result, because three interaction phenotypes would be apparent instead of two. The objective of this paper is to present the experimental data and our modified gene-for-gene analysis. Additionally, inoculation and genetic experiments designed to test the assumed gene-for-gene model were derived, and some implications of the model to breeding slash pine for fusiform rust resistance were considered.

MATERIALS AND METHODS

Host clones.--Individuals from eight full-sib families of P. e. elliottii were propagated by rooting cuttings. The cloned individuals were part of a study conducted the year before in which seedlings from 31 full-sib families were inoculated with 2 C. q. fusiforme cultures. Each cloned individual was infected during the previous study and had been saved for evaluations of gall development. At the time of initial propagation the donor plants were approximately 14 months from seed and had been hedged two times. Following a second cutting and propagation, 60 clones were represented by 4 or more rooted cuttings. The rooted cuttings from these clones were up-potted to 3.78-L pots and hedged to promote the regrowth of multiple, succulent shoots for subsequent inoculation.

Pathogen cultures.--Two single urediniospore derived cultures of C. q. fusiforme were used in this study, CCA-2 and WLP-10. The cultures were developed with the procedure described by Doudrick et al. (1993), which is a modification of Power's (1980) method for developing single aeciospore derived cultures. Both cultures originated from aeciospore collections made in 1984 from Livingston Parish (LA) loblolly pine (P. taeda)-- CCA-2 in Madison County, FL, and WLP-10 in Livingston Parish, LA.

Artificial inoculations.--The forced air apparatus of Snow and Kais (1972) was used to inoculate the rooted cuttings. For each cutting, the 1 to 7 (mostly 2 to 4) most succulent terminal shoots were inoculated at a density of 12 to 18 spores per mm². The inoculum density was verified after every tenth cutting inoculated. For each clone, two to eight rooted cuttings were inoculated with each culture. The cuttings were inoculated in random order, half the cuttings with CCA-2 and the other half with WLP-10, resulting in two completely randomized experimental designs.

Following inoculations, the rooted cuttings were incubated in the dark at 20 to 22 °C and 100% relative humidity for 24 hours. After incubation, the cuttings were returned to the greenhouse and grown under an 18-hour photoperiod provided by 1,000-W metal halide lamps. Two weeks after inoculation, the cuttings were fertilized once with 9-45-15 (liquid N-P-K) then

every second week thereafter with 20-10-20 (both, 200 ppm N). The presence or absence of fusiform rust galls were recorded 6 and 9 months after inoculation. For this analysis, a cutting was considered infected if at least one of the inoculated shoots was galled at the 9-month scoring.

Data analysis.-- To be included in the analysis, a clone must have had at least three rooted cuttings inoculated with each culture. Of the 60 clones inoculated, 19 met this criterion. A non-galled cutting was interpreted to represent the low infection type (L) of the union phenotype (Nance et al. 1992), whereas a galled cutting represented the high infection type (H). To account for variation between the infecting spores (basidiospores) within a clone:culture combination, we assumed that for a given host clone, any result other than 100% non-galled or 100% galled indicated heterozygosity for the culture's corresponding gene. Thus for each clone:culture combination, we observed one of three classes of union phenotypes-- all low (L), all high (H), or low and high (L,H)-- instead of the usual two classes (L and H).

Because clear interactions (i.e., infection type reversals) were evident between several pairs of clones and the two cultures, the infection type data were analyzed under the assumption of a gene-for-gene model (Loegering 1984). A modification in the analysis was necessary to utilize the three phenotypic classes. In the modified analysis, we assumed that both the L and L,H union phenotypes were definitive because L were observed in both, but different because H were observed in L,H only (Table 1). The resulting analysis procedure was as follows:

- (1) sort the clones into union phenotype groups;
- (2) try to fit the current model (initially a one CGP model) to a group;
- (3) if it fits, designate the culture and clone genotypes and move to the next group; if it does not fit then (a) add a CGP, (b) designate culture and clone genotypes, and (c) move back to previous groups and designate clone genotypes for the new CGP; and
- (4) move to the next union phenotype group and proceed with step 2.

RESULTS

Data for 9-month post-inoculation observations are summarized in Table 2. Based on the pattern of union phenotypes, the clones were sorted into like groups (i.e., outcomes). With three phenotypes ($u=3$) and two cultures ($c=2$) a maximum of nine groups (u^c) and four corresponding gene pairs [$(u-1)c, u=2$ or 3 and $c>1$] could potentially be identified. Eight of the nine phenotypic groups are represented. The only group not represented is low CCA-2 and low, high WLP-10 (L/L,H).

Results from our modified gene-for-gene analysis of the data in Table 2 are presented in Table 3. Given the 2 cultures and 3 phenotypic classes, the maximum number of CGPs were identified with this set of 19 clones. With these two cultures, the missing group (L/L,H), could not differentiate another CGP. However, its postulated genotype can be determined and it is shown in the last row of Table 3. (Note that if the L/H clone is homozygous for the definitive allele at CGP4 (1h4) and the H/L,H clone is homozygous for the definitive allele at CGP1 (1h1) then a cross of these clones would yield all L/L,H progeny). Also, the complete genotype of the H/H clones can be postulated (Table 3, first row), since the postulated genotypes of both cultures contain at least one definitive allele for each CGP.

The data analysis proceeded as follows: Observing that the H/H group provides no information, because the clones and the cultures could be either 0 or 1 at each CGP, we moved

on to the H/L,H group. Here we found the first CGP and designated the H/L,H clone 1h1³ and the CCA-2 and WLP-10 cultures 0p1 and 1,0p1, respectively. Moving to the H/L group, we first attempted to explain the data with CGP1. This simply involved trying 1h1 and 0h1 for the H/L clone. Neither possibility fit, so we added a second CGP and designated the clone ?h1 1h2 and the cultures 0p1 0p2 and 1,0p1 1p2. The clone genotype at CGP1 is designated ?h1 because it could be either 0h1 or 1h1. Moving back to the H/L,H clone, we observed that it must be 1h1 0h2. We then proceeded to the L,H/L,H group, continuing this procedure on through to the last group. Finally, the completed table contains postulated genotypes for both cultures and all the clones.

Table 1. Union phenotypes and associated host clone and pathogen culture genotypes for one corresponding gene pair.

Urediniospore (n+n)					
Culture Genotype		AA	Aa	aa	
Boolean Code		1p1p	1p0p	0p0p	
		↓	↓	↓	
Basidiospore (n)					
Progeny Genotype		A	A,a	a	
Boolean Code		1p	1p,0p	0p	
<hr/>					
		Union Phenotypes			
Host Clone		<hr/>			
(2n) Genotype	R_	L	L,H	H	
Boolean Code	1h	1u	1u,0u	0u	
<hr/>					
Host Clone					
(2n) Genotype	rr	H	H	H	
Boolean Code	0h	0u	0u	0u	

Notes: Gene symbols in the cultures are A (avirulent) and a (virulent) and in the clones R (resistant and dominant) and r (susceptible and recessive). The L and L,H union phenotypes are assumed to be definitive, thus the L union results from the R_ clone and the AA culture only and the L,H from the R_ clone and the Aa culture only. All other clone:culture combinations result in the H union phenotype, i.e., the non-definitive. The Boolean codes are adopted from Loegering (1978, 1984) and are useful for data analysis when more than one CGP is involved. The Boolean rule for determining union phenotypes and clone and culture genotypes is:

$$1h + 1p = 1u = L; \text{ and } 1h + 0p = 0h + 1p = 0h + 0p = 0u = H.$$

DISCUSSION

The application of a standard gene-for-gene analysis to these experimental data would have resulted in the detection of two corresponding gene pairs (CGP2 and CGP4). The clone:culture combinations producing both low and high infection types would have been discarded, attributing the anomaly to variation in inoculation technique or host tissue suitability. In the present study, we could not conclude that variation in technique or tissue suitability caused these results. Instead it appeared that the variation was caused by

³ The trailing number in the genotype and phenotype codes identify the CGP.

segregation of heterozygous loci for pathogenicity in the cultures. Inoculating multiple shoots of at least three rooted cuttings per clone with a moderately low inoculum density, evidently provided this opportunity. The fact that in only 5 out of 17 cases of low and high infection types (L,H union phenotypes) the percent galled was less than 50 percent reinforced our tenet that variation was due to segregation. A straightforward test of the tenet can now be proposed— that the same experiment run with a high inoculation density (e.g., 120 to 150 spores/mm²) would reduce to a two gene system (i.e., the L,H union phenotypes would convert to H phenotypes, while the L phenotypes would remain L).

Table 2. Summary of infection type data for 19 slash pine clones and 2 single urediniospore derived C.q. fusiforme cultures.

Clone	Family	CCA-2		WLP-10		Group
		#Gall/#Inoc	Interaction	#Gall/#Inoc	Interaction	
396	556	3/3	0	3/3	0	H/H
397	556	3/3	0	3/3	0	H/H
399	586	3/3	0	3/3	0	H/H
401	586	3/3	0	3/3	0	H/H
572	586	3/3	0	1/3	1,0	H/L,H
577	586	3/3	0	0/3	1	H/L
507	536	7/8	1,0	6/7	1,0	L,H/L,H
514	536	4/5	1,0	4/5	1,0	L,H/L,H
517	536	3/4	1,0	3/4	1,0	L,H/L,H
520	536	1/3	1,0	2/3	1,0	L,H/L,H
537	556	3/8	1,0	2/8	1,0	L,H/L,H
531	556	2/4	1,0	2/4	1,0	L,H/L,H
559	582	2/4	1,0	2/4	1,0	L,H/L,H
510	536	1/3	1,0	3/3	0	L,H/H
555	581	2/3	1,0	0/3	1	L,H/L
536	556	0/3	1	3/3	0	L/H
495	531	0/4	1	0/4	1	L/L
528	551	0/3	1	0/3	1	L/L
543	581	0/3	1	0/3	1	L/L
Totals		43/73		40/72		
		59 %		56 %		

Notes:

#Gall is the number of galled rooted cuttings.

#Inoc is the number of rooted cuttings inoculated.

Interaction is the union phenotype (Nance et al. 1992) using Loegering's (1978, 1984) symbolization, where 1 is definitive and 0 is non-definitive. A definitive union phenotype (L = 1u or L,H = 1,0u) results only from definitive host and pathogen genotypes (i.e., 1h + 1p = 1u or 1h + 1p,0p = 1,0u). A non-definitive union phenotype (H = 0u) results from all other combinations of host and pathogen genotypes (i.e., 1h + 0p = 0h + 1p = 0h + 0p = 0h + 1p,0p = 0u).

Group is a classification of the host clones according to their reaction to cultures CCA-2 and WLP-10, where L = low infection type (definitive), H = high infection type (non-definitive), and L,H = both low and high infection types (definitive and non-definitive).

Table 3. Postulated culture and clone genotypes for the four corresponding gene pairs detected in the modified gene-for-gene analysis.

			Corresponding Gene Pair (CGP)				
			1	2	3	4	
			Pathogen	Culture	Genotypes		
Group	Union Phenotypes	#Clones	CCA-2	0p	0p	1,0p	1p
			WLP-10	1,0p	1p	0p	0p
Host Clone Genotypes							
CCA-2/WLP-10	CCA-2/WLP-10						
H/H	0u/0u	4	0h	0h	0h	0h	0h
H/L,H	0u/1,0u	1	1h	0h	0h	0h	0h
H/L	0u/1u	1	?h	1h	0h	0h	0h
L,H/L,H	1,0u/1,0u	7	1h	0h	1h	0h	0h
L,H/H	1,0u/0u	1	0h	0h	1h	0h	0h
L,H/L	1,0u/1u	1	?h	1h	1h	0h	0h
L/H	1u/0u	1	0h	0h	?h	1h	1h
L/L	1u/1u	3	?h	1h	?h	1h	1h
L/L,H	1u/1,0u	0	1h	0h	?h	1h	1h

Notes:

Genotypes for clones and cultures are designated as follows: 1 = definitive, 0 = non-definitive, and 1,0 = both definitive and non-definitive. Union phenotypes are a function of the clone and culture genotypes across the CGPs. For example,

Culture	WLP-10	CGP			
		1	2	3	4
		1,0p	1p	0p	0p
Clone	H/L,H	1h	0h	0h	0h,

results in a 1,0 union phenotype (coded 1,0u1, where the trailing 1 indicates the definitive or expressed CGP), because the lowest infection type is "epistatic" to all higher infection types (Loegering 1984). In this example, for the segregation at CGP1 (1p) to be expressed, the interactions of CGPs 2-4 must all be non-definitive.

Some of the very high percent galled clone:culture combinations could be H union phenotypes instead of L,H, but this would not have qualitatively changed the outcome of our analysis, we simply would have had more clones in the H/H group and no clones in the L,H/L group. More problematic is the question of the non-definitive (H) union phenotypes. A failure to identify non-definitive phenotypes would certainly lead us to reject the gene-for-gene hypothesis. Currently, we have little power to determine the correctness of the H phenotypes, and, in several of these cases, fewer than half of the inoculated shoots were galled. However, we have observed substantial morphological variation among shoots within rooted cutting ramets of slash pine and it is generally thought that this variation is correlated with host tissue

suitability for infection⁴. Thus, it may not be surprising to find H union phenotypes with less than 50 percent galled shoots. Also note that the same conclusions and problems apply regardless of whether the low or the high infection type is the definitive.

Assuming that we have correctly classified the infection types, the postulated CGP genotypes of the clones and cultures should be valid. Genotypes postulated in this manner have proven to be at least as useful as those derived from genetic studies (i.e., inoculating polymorphic parents, and their F1 and F2 progeny) (Loegering and Burton 1974). However in either case, the postulated genotypes must be verified by studying the inheritance of the CGPs in controlled crosses of the clones and the cultures. For example, selfing an H/L,H clone and inoculating rooted cuttings of the progeny with WLP-10 would determine whether or not the clone was homozygous or heterozygous for 1h1. If the clone is heterozygous and the union phenotypes segregate 3 L:1 H, then resistance is dominant to susceptibility. Likewise, selfing WLP-10 and using clonal lines (single urediniospore derived cultures) of the progeny to inoculate the H/L,H clone should result in a 1 L:2 L,H:1 H ratio of union phenotypes. Many genetic tests can now be proposed by simply observing the postulated genotypes presented in Table 3.

The results of this study suggest that gene-for-gene specificity exists between slash pine and the fusiform rust fungus. With only 2 pathogen cultures and 19 host clones, we have hypothesized the presence of 4 genes controlling gall formation on slash pine rooted cuttings. That these genes control specificity during the initial point of interaction, suggests that they may have a major affect on the genetic structure of the pathogen population. Genes controlling different forms of specificity will no doubt be found, resulting in less severe consequences for the pathogen population. Arguments for finding and using the later type of genes in resistance breeding programs are good (Snow et al. 1975; Powers and Matthews 1979), because adequate resistance is accomplished without "forcing" the pathogen to mutate (Loegering 1984; Mundt 1990). Sources of resistance that place little selection pressure on the pathogen population will no doubt be more durable than the type of resistance investigated in this study.

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CLOSING SESSION

GENETIC DIVERSITY IN CONIFER STANDS: EVALUATION, MAINTENANCE AND IMPROVEMENT

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Abstract. --Maintaining an "optimum" level of genetic diversity is often espoused as a reason to avoid plantation forestry as it is commonly practiced in the Southeast. Natural regeneration with selective harvesting is expected to yield a more diverse population than planting with seed orchard based seedlings and clearcutting. Whether or not this philosophy is valid is dependent upon the measure of diversity, the level of diversity in the originating population, seed dispersal patterns, reproductive phenology, natural selection, genetic drift, etc. Small, patchy highly related groups of trees are possible in naturally regenerated forests and successive generations may become inbred or extremely narrow in their genetic base. Effective population size, coancestry levels, and heterozygosity levels are all valid measures of genetic diversity, but each must be used in the proper context. As we attempt to discuss genetic diversity we must consider "what we want", "what we mean", and how we are going to measure it.

Keywords: Pinus taeda, effective size

INTRODUCTION

Increasing interest in the basic ecology of our forests has led to concerns about their ability to adapt to changes in the environment, their response to pestilence and other disasters, and the general population structure. Maintenance or enhancement of genetic diversity has become a driving force in many land management decisions and will continue to impact the forestry profession for many years. Genetic diversity has been used to describe the number and frequency of plant species that occupy a given area. It has also been used to describe the variability within a given species within a given area (El-Kassaby, 1992; Ledig and Conkle, 1983). The term means different things to different people. A commonly held belief among the general population is that natural systems promote diversity and artificial systems retard it. This belief stems from common selection and breeding practices whereby a sample of a population serves as the parents of the next generation. Agronomic crops such as corn and wheat demonstrate the potential of breeding programs to narrow a population's gene pool. Forest tree breeding is often viewed as synonymous with agronomic crop breeding and a rapid and perhaps permanent loss of the natural population diversity is cautioned against. Determining the change in genetic diversity in a forested system as a result of forestry activity can be a simple task or a difficult if not impossible endeavor. Simply noting the changes in the frequency distribution of species after a particular forestry activity is a fairly simple task on a relatively small area. Stands that are converted from mixed pine hardwood to pine plantation show obvious changes in species diversity. However, documenting the number of genotypes for a given species that occur in a section forest is a formidable task.

In natural pine stands that are being harvested and replanted with improved nursery stock, a reduction in genetic diversity within the given species might be assumed but might not necessarily be correct (El-Kassaby, 1992). Quantifying the genetic diversity within a pure stand of a single species is much more

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difficult than merely enumerating the species that are present in a multi-species stand. Discussion of genetic diversity relative to forestry activities requires a base line that accurately describes the reference point. Given an accurate reference point it is also essential to know what level of diversity is optimum or desired for a given situation. In other words you must know where you are starting and where you are going in order to decide what mechanisms are proper and what mechanisms are detrimental.

WHAT DO WE MEAN?

Genetic diversity can refer to the number of different genotypes that are present in stand or the level of heterozygosity that is present or the level of coancestry among the trees. The number of genotypes in almost any conifer plantation, either naturally or artificially regenerated, is virtually equal to the number of trees. The exception being clonal or vegetatively produced seedlings which all share the same genotype. Replacing 1000 trees from a natural stand with 1000 trees from seed orchard stock would not reduce the diversity level because they would still be 1000 genotypes. Measuring diversity in terms of heterozygosity requires specification of the loci to be considered and then careful analytical work to determine the genotypes at those loci. Using this measure may produce some surprises. It is possible to replace a 1000 tree natural stand with seed orchard stock and to increase the heterozygosity level. Naturally reproducing stands often have family structure and perhaps even some genetic drift effects which reduce heterozygosity levels. Seed orchard stock from a fairly large number of non-related parents could easily have a greater degree of heterozygosity.

Coancestry is another measure of diversity. In other words, what is the average relatedness of the individuals in a given area. The contrast with natural stands will depend on the size of the stand. Small stands generated from a relatively small number of parents may be highly related and actually have a lower diversity than the seed orchard stock. As the size of the area is increased the average coancestry would be reduced as more and more families were introduced. At some point, the coancestry of the seed orchard stock would be less, because it remains fixed and the natural stand level is decreasing.

Effective population size may also be used as a measure of diversity. The effective population size will be dependent on the number of parents, the family structure of the parents and their reproductive phenology. As with coancestry levels, the effective population size of a small stand may be much lower than that of the seed orchard stock. One or two trees may be dominating the gamete pool due to their fecundity (El-Kassaby, et al, 1989), their phenology (Askew, 1986), or their method of seed dispersal (Schuster and Mitton, 1991). As the stand size is increased, more and more parents will be represented in the gamete pool and eventually the effective size will be greater than the fixed level of the orchard stock.

So the question "What do we mean?" is important. Any discussion of genetic diversity requires a specified measure and a common understanding of that measure. It may be possible to equate two stands using one measure and to find them vastly different using another. Both measures would be correct and both would have valid interpretations when used in the proper context.

WHAT DO WE WANT?

Commercial forestry concerns need a steady, reliable supply of wood. Pulpwood and sawtimber demands keep rising as the available land resource continues to shrink. Growing more wood on less land requires continued improvements in both the growing stock and the cultural techniques. Improvement in the growing stock necessarily involves some form of selection and perhaps some form of breeding. Selecting the fastest growing trees and the trees with the best characteristics such as form, specific gravity, disease resistance, etc. may require a breeding population that has only a fraction of the base population represented. The number of potential parents involved in any one breeding program is affected by the breeding strategy, the available area for breeding and

testing, and the breeding technique. Open-pollinated orchards generally can utilize a higher number of parents than breeding programs that rely on controlled crossings. Breeding programs that seek to perfect a given trait may be extremely limited in their breeding size due to cost of screening, effectiveness of screening, or the proportion of trees with the desirable trait. Improvement of a population mean necessarily requires a shift away from the less desirable trees to the more desirable trees. Will the shift eliminate too much of the genetic variability? If the program is completely successful it will eliminate virtually all of the genetic variability in the trait to be improved, but what about the non-selected traits?

Forest geneticists and the public in general are concerned about the maintenance of a broad genetic pool in all species in order to provide the adaptability that will be necessary to meet the changing environment (Millar et al, 1990). Where possible, natural systems are being preserved and even recreated by reintroduction of species that have been eliminated. As forest genetics work progresses will the genetic variability that we seek to maintain be reduced to an unacceptable level? What level is unacceptable? How will we know when we get there? What is the most rational approach to maintaining the gene pool in the species that we exploit for commercial gain: Seed banks, clone banks, DNA banks? What should we be doing about the species that are displaced or constricted when commercial species are favored? Do we need to conserve the gene pools of all species?

HOW DO WE MEASURE IT?

For the most part, answers to the questions "What do we mean?" and "What do we want?" are dependent on our method of measure. Genetic diversity is usually discussed in terms of species richness, species frequency distributions, number of species etc. Genetic diversity can be discussed in quantitative terms such as coancestry, inbreeding coefficients, effective population size, and heterozygosity levels. But it can also be discussed in terms of sustainability. That is, how well can the population be expected to maintain itself through time. If there is no interference with the population can you expect it to reproduce itself? Will the population become fragmented and less diverse due to drift? Will other populations migrate into it or will it emigrate into other populations?

It is reasonable to consider whether or not quantitative measures such as heterozygosity levels are correlated with performance levels. Does increased heterozygosity imply increased stability (Ledig and Conkle, 1983; Strauss, 1987). Will a population that has an equal distribution of 3 alleles for a given isozyme be measurably different in any morphological, physiological, or empirical traits from a population that is homozygous for any one of the alleles? These questions are important because we do not strive to maintain a number that is meaningless in practical terms of population dynamics. The idea that more is better may be true for genetic diversity but there needs to be some evidence that it denotes a healthier more adaptive population. Linkages and gene effects need to be documented. The adaptive ability of conifers relative to their measured heterozygosity using enzyme markers or DNA analysis is not well established. The amount of the gene pool that is lost by selection practices is impossible to determine. Inbreeding effects can be surmised using basic theory to estimate the loss in heterozygosity, but selection practices most often do not directly result in inbreeding and the degree of inbreeding in a given population is rarely known.

Effective population size is a reasonable measure of the relative fitness of a population. However, many factors affect the measure. The number of unique genotypes is one factor, but the role of reproductive phenology and fecundity are very important in many instances and germination and seedling survival must also be considered. Also, knowledge of the geographic position of the trees is vital to understanding their interaction potential. Fecundity, phenology and germination percentage may change each year or work in some cyclical fashion and the effective population size will fluctuate as these factors change. Effective population size is measure that is not stable and is only a snapshot of the situation.

Any measure is only meaningful relative to some standard. A high level of genetic variation in one instance may be low in another. Small isolated naturally occurring stands of trees may have a severely restricted gene pool. Replacing them with seed orchard stock may provide a doubling of the variation. That same level of variance may be a step backward if the original stand is vast and not well structured (genetically). Increasing the number of parents may be an improvement by one definition but if the parents are not well suited for each other in terms of reproduction, the stand will not be stable and may quickly develop into small inbred, isolated stands with poor variation within the stands and perhaps great variation between them.

With all of the choices for measuring diversity, which one is ideal? All have their application and all could be considered ideal for a given situation. For the forest geneticist the question is usually "Will the breeding and selection program produce a population that is too restricted?". If the question pertains to the outplanted population that will be produced from the breeding material, the entire gamut of reproductive characteristics and pedigrees must be considered. Open-pollinated seed orchards produce seed that have genetic qualities imparted from the parents and weighted by the parental contributions. Measurements of reproductive phenology, fecundity, germination potential must be included along with the parental pedigrees. In this case the effective population size is a reasonable estimate of the potential genetic diversity to be imparted by the breeding population to the outplanted population. If the breeding population alone is to be considered, pedigrees and coancestry coefficients may be sufficient.

Regardless of the measure of diversity, a basis of comparison is still necessary. If we anticipate a 50% reduction in heterozygosity, is it too much? What is a proper balance between selection differential and diversity.

Is it reasonable to use a "natural" population as a basis for comparison? Are most natural populations structured without any influences from development or from logging, high-grading, or other land manipulation? Should each breeding program be expected to incorporate all of the base population's diversity into its selected parents? If it were to do so the selection differential would be 0. How should we proceed? Complete improvement completely eliminates diversity for the trait and complete diversity eliminates improvement. Moreover, how many traits must be considered when measuring diversity. By including a sufficient number, the diversity measure would be imperceptibly changed even when one or two traits were completely fixed or lost.

It is obvious that there are more questions than answers and sufficient ambiguity to allow for justification of any stance. In the mean time, breeding and selection must proceed and new methods of improvement i.e clonal forestry, gene technology, etc. will still further complicate the questions. It is in our own best interest to establish some working definitions and guidelines for conserving the gene pool in our forest species. We need measures of diversity that are both meaningful and calculable. Most importantly, we need to understand the implications of our breeding and improvement systems.

HOW DO WE ACHIEVE A BALANCE?

In order to determine the impacts of forestry activities and genetic improvement programs a solid scientific determination of the species' genetic diversity must be developed first. Major commercial species such as loblolly pine (*Pinus taeda* L.) need to be surveyed to document as much as possible about their range-wide diversity. Linkages between traits of interest for improvement and other more basic traits such as reproductive phenology should be determined so breeding system implications can be estimated.

Deployment strategies for orchard derived material or vegetatively propagated material can be designed to maximize use of the available genotypes

in the mix. The question "How many clones?" was asked several years ago (Libby, 1980) and is still a viable question. The answer must include the level of diversity that exists within the clones. Use of uneven age management systems may allow for a better mix of genotypes by planting smaller groups within a given management unit and then spreading the total unit replacement over a longer period of time. Multi-species management may also be viable alternative if the productivity level can be maintained and the cost does not become prohibitive. Diversity within a given species may be reduced by the breeding program but the species diversity may be greater than would be maintained with a single species approach.

Breeding populations must kept as broad as possible. As we continue to select for desirable traits we must make every effort to include as many genotypes in the mix as possible. Mating designs must be designed to prevent rapid narrowing of the population and coancestry levels must be considered in all schemes. Before we can formulate methods of maintaining or improving genetic diversity we must develop sound, meaningful, realistic measures of diversity that can be used to evaluate what already exists and to examine the implications of proposed management systems.

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ALLOCATION AND MANAGEMENT OF GENETICALLY IMPROVED STOCK: A "MISSING LINK"

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Abstract.--Tree Improvement programs worldwide have devoted significant time and resources, quite successfully, to the development of varieties of commercial trees with substantially improved value potential. However, guidelines and procedures to assure maximum realization of the value of such varieties in operational forestry are seldom discussed, despite the significant opportunity costs and risks which can be incurred when such guidelines and procedures are lacking. Weyerhaeuser Company plants over 20,000 ha. annually to genetically improved seedlings of coastal Douglas-fir, Ponderosa and lodgepole pines, western hemlock and Noble fir on its western timberlands, and during the past two years, detailed standard guidelines and procedures have been developed and implemented to guide the characterization of families, the allocation of families to planting sites, and the tracking and monitoring of improved stands through time. From this experience was gained an understanding of the critical factors leading to successful implementation of genetic improvement in field forestry. These factors will be articulated, and supported by specific examples taken from Weyerhaeuser's Douglas-fir program. The benefits, expected and unexpected, from a well-planned and successful allocation system have been great, in consistent effective use of stock, in control of risk, in defensibility of genetic improvement practices outside the Company, and in improved communication and coordination between Tree Improvement and operational foresters.

Keywords: Pseudotsuga menziesii, realized gain, allocation, regeneration, seed source movement, genetic diversity, inventory, family blocks.

INTRODUCTION

The success of most genetic improvement programs is reflected in the changes which are realized in the attributes of the target species or in the products derived from those species within an operational production system. In the case of commercial forest trees, that operational production system is typically some type of planted and managed forest stand, from which a stream of products is harvested. Genetic improvement programs for commercial trees have focused the greatest time and energy to date on the development of varieties with improved productivity, stem form, wood quality, disease resistance,

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and/or hardiness to cold or drought; on propagation systems for 'packaging' of the improved genetic potential; and (particularly in recent years) on gene conservation activities supporting the long-term breeding program (Figure 1). This focus on varietal development has been very successful in terms of the potential it has created for improved value on the ground. However, with few exceptions two critical steps have seldom been discussed, steps involving effective allocation of genetically improved stock to operational planting sites, and effective utilization through optimal silviculture and processing. Lack of attention to these steps can mean loss of much or all of the value potential created in the selection, breeding and testing process. On the 'up-side', there is an enormous potential for additional value to be created in allocation and utilization. This added value can come from proper matching of genetic material to site and market requirements; from nursery management and silviculture which enhances positive attributes of the genetic materials or corrects negative attributes of those materials relative to site and market requirements; or from coordination in planting of specific varieties with design and location of processing facilities to ensure a consistent supply of a desired type of raw materials for target products, to name just a few. Experience and observations of a large number of programs indicates that the value impact of tree improvement is more limited by the ability to *deliver that value on the ground* (through allocation and utilization) than it is by the ability to improve *genetic potential* in selection, breeding and testing.

Allocation of Genetically Improved Stock - Definition

In the context of this discussion, the term *allocation* is employed to mean *the process of choosing planting stock for a site and deploying it on that site*.

Common Reasons for Loss of Gain Potential in Allocation

The effectiveness with which gain is realized through allocation is sometimes limited by organizational factors, sometimes by inefficient methods, and very often by conservative assumptions or risk-avoidance mechanisms which are applied more broadly than may be required. Some of the most common features of allocation processes or approaches that limit the realization of gain are:

1. Vaguely defined improvement goals or trait requirements.

It is very difficult to achieve an objective which has not been clearly articulated. However, it is an endemic problem in tree improvement that its goals apply to a timeframe which is decades out in the future, market and product definition in that timeframe is uncertain, and therefore trait requirements are uncertain. This problem is often confounded by the common situation that mill trial data or simulation models relating tree characteristics to product recovery and value are often limited in scope or lacking entirely, and geneticists often feel poorly qualified to expand these data and models (not to mention lacking the resources to do it). Despite these challenges, truly effective

breeding programs and allocation systems must be based on clearly defined trait-improvement goals. Without such clear goals, it is difficult to obtain sufficient resources to get the job done well, resources are wasted in activities which do not further the goals, and decisionmaking often becomes highly conservative - no future scenario is pursued intensively for fear that the (unknown) right option will be lost in the process.

2. 'Generic' varieties, 'generic' allocation

When trait requirements are not clearly articulated (for the reasons described above), it is most common for tree improvement programs to be designed to produce a single 'generic' variety which provides at least some economic benefit for any probable end-use and site type (within geographic constraints) but optimizes benefit for no site or end-use. This approach, while conceptually and logistically simple, has a large opportunity cost associated with it, and in fact may never produce a sufficiently dramatic result to maintain long-term support for the tree improvement enterprise.

3. 'Zone' based management of the risk of maladaptation

Most tree improvement programs were initiated based on the appropriately conservative premise that the risk of maladaptation is best controlled by selecting, testing and allocating genetic material within a delineated geographic area - a 'breeding zone' or 'seed zone'. As more programs complete one or more generations of testing, it is becoming apparent that, in many regions of fairly mild climate, family and site variation within these zones is as large or larger than zone-to-zone variation, both for value traits and for adaptive traits. When this is the case, the 'zone' approach to allocation not only does not provide good control of adaptive risk, but also unnecessarily limits the selection intensity and gain available from broader allocation of tested, proven genetic material from a similar environment but a different zone.

4. Genetic value predictions which are not comparable across all available materials

Most tree improvement programs have conducted their genetic testing in stages where different parents are tested in different series over a number of years. Often within a year there are different 'sets' of parents, only intercrossed within a set (disconnected diallels are an example of this). Second and third generation testing is now underway. Methods must be put in place (preferably at the mating and test design stage) so that comparable genetic values can be calculated across sets, years and generations, and so that genetic values can be compared for all family types which could be produced and allocated (wind-pollinated vs. SMP vs. control-pollinated). In addition, these genetic values must be well-understood by, and in the hands of, the people responsible for ordering and allocating planting stock for operational regeneration. Without such a system, the selection intensity available in selection and allocation can be considerably limited.

5. 'Stand-level' vs. 'landscape-level' requirements for genetic diversity

It is most often assumed that the risk of loss due to unforeseen genetic responses is best controlled through allocation of many families to any one planting site. On highly stressful sites or on sites where some type of highly heritable, density-dependent pest response is involved, this strategy is quite sound. Likewise, sites which are to be naturally regenerated in the next cycle should be planted with many families to minimize future inbreeding. However, this strategy passes over the very powerful knowledge base which can be gained through single-family block planting and monitoring through time - knowledge which can be applied directly to control of future risk, through elimination of certain families or adjustment of allocation rules. Single-family block planting and monitoring can also enable greatly improved targeting of certain families for particular site types, silvicultural regimes or product directions as operational experience is gained over a period of years. As long as many families are planted across a geography and the proportion of planting to any one family is controlled (i.e. 'landscape-level' diversity), the risk of significant loss to some future genetic response under many situations may actually be decreased via operational monitoring of single-family plantings.

Key Elements of an Effective Allocation System for Improved Stock

First, what are the criteria for an 'effective' allocation system? In general, an allocation system should provide a logic and a process for effective decision making about what genetic material should be used in what situation; it should provide for management of risk - both the risk of maladaptation under the current range of conditions and the risk of loss due to unforeseen, future genetic responses; it should provide mechanisms for tracking success and improving the allocation rules/procedures; and it should facilitate sharing of information and building of synergy among the groups responsible for developing, propagating and using the genetic material. Based on these criteria, certain elements can be listed as critical components of an effective allocation system:

** A process for describing trait requirements: Preferably one which is tied to specific tree-growing strategies, end use requirements and site features.*

** A means to describe genetic material in terms of those requirements: In a manner which enables comparison of genetic values across years, sets, generations and available family types (wind-pollinated, SMP, control-pollinated).*

** Unambiguous, documented rules for choosing genetic material for sites: These must be well-understood and accepted by foresters, with a mechanism in place for the foresters to feed back and improve the rules. The more specific the matching can be of families with sites or end-use requirements, the more effective will be the utilization of the full range of families and sites planted.*

Key Elements of an Effective Allocation System for Improved Stock (continued)

** Specific prescriptions for management of risk: These should encompass all aspects of risk, including the risks of loss due to maladaptation or unforeseen future genetic responses, market risk or political risk.*

** Quantifiable goals, and processes in place to track success: These goals might be expressed in terms of the actual performance and losses realized in operational stands as compared to some specific baseline (such as stands of 'local' unimproved stock).*

** Mechanisms for feeding back and responding to operational-stand performance data: This requires that the genetic identity of stands be recorded, that some kind of systematic monitoring of survival, adaptive performance and value characteristics be carried out, and that someone has the accountability to summarize that information and report it back to the people responsible for propagation and allocation.*

** A strong formal and informal structure for information flow: The more people in the process who are sharing information about the genetic material, the greater will be the enthusiasm and synergy among those groups and the steeper will be the increase in efficiency and effectiveness of the production and allocation systems through time.*

In the next section, an example of an operational allocation system for improved Douglas-fir will be described, and some of the key learnings from the development and implementation of that system will be reviewed.

A CASE STUDY

Background

Since the mid 1980s Weyerhaeuser Company has been meeting its 20,000 ha/year annual low-elevation² Douglas-fir planting requirement in western Oregon and Washington with seedlings from first-generation, tested seed orchards. Company lands in this region are generally mild and highly productive, but are associated with a high degree of local variation in temperature, soil moisture, soil productivity, animal damage, vegetation competition and other factors. Sites also vary in operability, ease of management, and distance from particular market centers. Certain sites have been found to be poorer than others in expression of stem defects and other quality characteristics.

Douglas-fir is a species which is grown primarily for processing into solid wood products (i.e. lumber, beams, etc.), and is used most often in applications where product strength and stiffness and dimensional stability are required. Different geographies em-

2. Below 2000 feet.

phasize different products and markets, and a growing information base is becoming available on the relationship between site characteristics, raw material characteristics, and value for these products and markets. Weyerhaeuser is moving toward a more 'prescriptive' approach to forest management in each geography so that limited resources (forest management dollars, people time, genetic materials) can be put to their highest use.

Historically, Weyerhaeuser's Douglas-fir genetic material was developed, propagated and allocated within 6 'low-elevation' and 6 'high-elevation' breeding zones. Wind-pollinated orchard family mixtures containing 15-30 parents were used for operational planting. In the early 1980s, a series of broad-based research trials were installed to evaluate the stability of first-generation family performance across a wide range of environments both within and across breeding zone boundaries. A variety of hardiness and growth rhythm traits have been assessed in these trials along with growth performance, survival and frost damage in the field. A comprehensive review of the results of these trials was completed by Stonecypher in 1990. In summary, these trials show stable superiority of most families across a very diverse range of environments, including sites in 'non-local' breeding zones, and variation associated with sites and families within zones was considerably larger than zonal variation, for growth performance, growth rhythm and frost hardiness traits. These results likely reflect the mild selective environments and the 'fine-grained' pattern of site variation within Company ownership in low-elevations on the west side of the Cascade range.

In 1991, an effort was initiated to review, update and document operational allocation rules and procedures dealing with genetically improved Douglas-fir. A team was assembled representing the forestry operations, the nursery and orchard groups, tree improvement and forestry research, and the result of this team's work was a system for allocation which meets the criteria for effectiveness described above, and which is now implemented across the Company's low-elevation landbase.

Weyerhaeuser's Allocation Process for Improved Low-Elevation Douglas-fir

The allocation process consists of 5 principal steps, and these will be reviewed below.

1. First, planting units to be regenerated are classified in terms of their target market, their value potential, the operability and manageability, and their biological risk factors (cold, drought, defect potential, etc.). This is done by the foresters during the summer and fall, after site preparation, based on a standard worksheet and scoring system which uses the soil survey, physiographic characteristics, and local-stand characteristics. Planting units can then be ranked within and across geographies for value potential with key biological risk factors flagged out.

2. In parallel to the planting site classification process, genetic test data are summarized in the form of breeding values for growth potential, defect potential, wood specific

gravity and adaptive traits, and these breeding values are provided to foresters in the form of a catalog. Breeding values were standardized across years, sets and generations using a standard, unimproved check which was represented in all tests, and by scaling breeding values to an average variance derived as a pooled value from all tests. Defect potential, wood quality and adaptive traits are expressed as '+', '0' or '-' relative to the 'local' non-select.

3. A detailed 'rule-book' was written to guide the selection of families for planting sites based on the best possible match of growth quality and adaptive features:

- * Combinations of 'stress-sensitive' families and high stress-risk sites are to be avoided.
- * Combinations of defect-prone families and high defect-potential sites are to be avoided.
- * Positive adaptive or value traits of certain families are matched to sites with a particular requirement for such traits, due to a biological risk factor or a target market.
- * Otherwise, the fastest-growing families (highest volume breeding value) are allocated to the most productive sites.

However, extra safeguards are applied in the rules:

- * Limits are placed on the maximum 'environmental distance' from parental origin to planting site. 'Environmental distance' is expressed in terms of elevation, latitude, soil moisture availability and distance from the coast.
- * Limits are placed on the maximum proportion of planting to any one family, in a single year and over a 5-year period.
- * The contiguous area which can be planted to any one family or relative is restricted.

4. Families are allocated to planting sites as single-family lots, except on very stressful or extreme sites where balanced multi-family mixtures are prescribed.

5. The genetic identity of all units is tracked through the Company inventory system, a subset of units of each family is monitored annually, and the monitoring results are summarized and fed back to the foresters via the family catalog (described in #2, above).

The cross-disciplinary team which assembled to design the allocation process now has assumed a long-term review and improvement role, makes decisions involving use of genetic material for the highest overall benefit to the Company, and provides an important communication and approval mechanism relating to genetic improvement initiatives.

Key Learnings from the Allocation Process to Date

- * Clear specification of planting stock requirements and family attributes has proven to be extremely powerful. Not only is the most effective use being made of the wide variety of available genetic material, but no one has to trade off volume improvement for quality or adaptive trait improvement unless their sites or markets require it. The interchange of information which occurs during the specification process feeds information back into the breeding program about new trait priorities, and the process has generated a high degree of enthusiasm and involvement in tree improvement among foresters.
- * Management of families in single family lots in the orchard, nursery and field creates a very rapid learning curve, and much more knowledge than mixed-family management allows. Nursery cultural regimes and family-site allocation rules are already being revised based on operational experience with particular families. It is critical, however, to have a database structure ready ahead of time to accept and process this information and get it into the right hands for process improvement.
- * It has been extremely valuable to get the maximum amount of family information out into the hands of the orchard managers, nurserymen and foresters. Each of these people has become a more effective decision maker in management of their improved stock through the availability of this information, and they also become sources of important observational data.
- * Focused attention is required on the implementation of an allocation system so that the new rules and procedures become an integral part of the way people do business - *who* does *what* and *when*. Implementation requires clearly defined assignments, written into performance criteria, and training up and down the organization to ensure consistent understanding and support. This has proven to require enormous time, energy and persistence, even in the presence of a generally supportive atmosphere.

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GENETIC TEST RESULTS FROM A TREE IMPROVEMENT PROGRAM TO DEVELOP CLONES OF LOBLOLLY PINE FOR REFORESTATION

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Abstract. Eleven years ago, International Forest Seed Company began a tree improvement program to develop a population of loblolly pine (*Pinus taeda* L.) to resist infection by fusiform rust [*Cronartium quercum* (Berk.) Miyabe ex Shirai F. sp. *fusiforme*] and to improve volume production as well. One hundred and twenty-seven parent trees were selected from a pool of tested first-generation selections, then mated at random using small disconnected factorials. Seedlings emerging from rust screening with no rust galls were planted into cutting orchards, from which cuttings were taken, rooted, and planted into clonal field trials. Analysis of fifth-year data between seedlings and rooted cuttings of the same checklots reveals that there are no propagation effects. Further, rooted cuttings of the select clones performed significantly better than the commercial checks in terms of morphological traits and improved resistance to fusiform rust. Genetic analysis reveals a strong clonal effect and a possible G x E interaction for the morphological traits evaluated. Sensitivity analysis on the error variance of the clone mean suggests a reallocation of resources in designing future clonal studies.

Keywords: *Pinus taeda* L., fusiform rust, rooted cuttings, clonal propagation

INTRODUCTION

International Forest Seed Company (IFSCO) initiated a loblolly pine (*Pinus taeda* L.) tree improvement program in 1982 to specifically develop a population of highly rust resistant clones. Based on results of informal surveys and queries of forest land owners, fusiform rust [*Cronartium quercum* (Berk.) Miyabe ex Shirai F. sp. *fusiforme*] was sufficiently serious at the time to justify a tree improvement program with the aim of improving the resistance to the pathogen. Joining the North Carolina State University-Industry Cooperative Tree Improvement Program, provided IFSCO with the base population of tested trees from which superior selections were chosen to breed. Rooted cutting procedures used by Hilleshog AB, Landskrona, Sweden were the basis of the system applied at IFSCO to clonally propagate the offspring population. The purpose of using clonal propagation was to capitalize on both the time savings to large-scale implementation and the relatively larger genetic gains available through a clonal tree improvement program.

THE PROGRAM

One hundred and twenty-seven parent trees were selected from a pool of tested, first-generation selections from a combination of two sources: North Carolina State University-Industry Cooperative Tree Improvement Program, and the Cooperative Program between the USDA Forest Service and the Georgia Forestry Commission. Traits for parental selection include superior resistance to fusiform rust and superior height growth as evidenced in progeny tests. The select trees were mated in small disconnected factorials (generally 4 x 4) from 1983 through 1985.

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Seedlings from each successful cross were grown at the USDA Forest Service, Bent Creek Resistance Screening Center and screened for infection of fusiform rust using standard techniques (Anderson *et al.* 1983). Seedlings emerging from the screening with no rust galls became the base population for field testing. In some cases well over a hundred seedlings from a cross were rust free. All were planted in the cutting orchard, but in the later stages of field testing, a maximum of only 25 seedlings per cross were tested.

The cutting orchard was established by planting the six-month-old rust-free survivors in double rows with 6 ft² of growing space. Within six weeks after planting, the seedlings were initially hedged. Thereafter, the seedlings were hedged three times per year. Hedging served two purposes: (1) to maintain juvenility (Libby *et al.* 1972) and (2) to increase the number of potential cuttings (Foster *et al.* 1981). Other cultural practices included drip irrigation, pesticide applications, fertilizations, and weed control. Each practice was conducted on a very regimented basis, because we found that the hedge health had a tremendous influence on rooting success.

Initially, two populations of clones were to be maintained: (1) a breeding population and (2) a production population (Foster 1986). The purpose was to begin propagating the breeding population by selecting the best 300 clones after only three years of field testing. By age five only 160 were to be kept for breeding. This early selection procedure expends effort in maintaining some clones that would have never been included in subsequent generations, but shortens by two years the time required before crossing can be completed. The production population basically followed the same strategy of making more initial selections (in this case 200) than what ultimately was to be the final population (50 clones).

Currently, only the production population is being employed to any degree of work. A few thousand have been established in limited reforestation, primarily as demonstration studies. Some breeding population selections have been made, but propagating the clones and preparing them for mating has stalled. This is due to the apparent lack of market demand for this population.

PROPAGATION PROCESS

Since the program began, several improvements and modifications to our propagation procedures have been made. The objective always has been to produce large quantities of high quality rooted cuttings at reasonable costs.

Originally, after the seedlings were planted in the cutting orchard, the bud was nipped to retard height growth. Individual hedges were maintained at 1.5 feet via very selective hand pruning. When hedges are established now, the seedlings are allowed to grow up to three feet (12 months of growth) before they are hedged. This practice results in larger trees with many more acceptable cuttings (100 to 150 per plant). The hedge health is much easier to maintain as well. Good quality cuttings can be taken three times per year: in March, June, and October. After cuttings are taken, the plants must be hedged severely and the dead thatch removed.

Cuttings taken are best when they are three to four inches long with emerging needles at least 1.5 inches long. Only in October is a terminal bud necessary. They are cut to length during collection, placed in plastic bags and stood upright in cold storage approximately for three to seven days. Plant growth regulators involved in the rooting process are basipetally transported and accumulate at the base of the cutting. All cuttings are set directly to the cavity in which they will root and grow, after each cutting is dipped into IFSCO's modified Hare's rooting powder (Hare, 1974) that was developed to promote rooting. The cavities (5.6 in.³) are filled with a peat:perlite (60:40) mixture.

The 40 cavity trays are set in a greenhouse equipped with specific equipment to maintain 95 to 100% relative humidity. This is primarily accomplished with a fogging machine. Air temperatures are held close to 85 degrees F, while soil temperatures are maintained at 85 degrees F. During the winter this is accomplished with infrared heat. It takes approximately 12 to 14 weeks for rooting to

occur. The goal during this period and the following three months is to lay down root growth, but inhibit shoot growth. We found our highest rooting percentages to occur in the fall.

IFSCO, during the course of producing cuttings for this program and other studies, has set in excess of 700,000 cuttings.

FIELD TESTING

Experimental Design

From 1986 through 1990, a total of ten clonal field tests were established across the Coastal Plain of the Southeastern U.S. For the purposes of this paper, we will focus on the five tests that are at least five years old. These tests are located near the towns of Blakely, Claxton, and Dublin in Georgia; Excell, Alabama; and Walnut Hill, Florida.

In order to further screen the greenhouse-test survivors for fusiform rust resistance, each test was located in a high rust hazard area (Anderson *et al.* 1986). A high hazard site was defined as an area where neighboring forest stands exhibited over 50 percent infection. Geographically diverse tests in high rust hazard areas are intended to maximize sampling of divergent rust strains and promote selection for generally resistant clones.

The field tests were designed to achieve a compromise between (1) the number of clones that could be tested given a fixed number of trees that could be planted and (2) efficiencies of genetic value estimation for families and clones within families (Shaw and Hood 1985). Each tested clone was planted at each location with two ramets per clone per location, providing a total of ten ramets per clone (five locations x two ramets per location). At each location, one ramet per clone was placed in each of two complete blocks with clones from a single family distributed among six blocks per location. This design achieved balance at the family level across replications and locations, but was only partially balanced at the clone within family level. Clones from single families were distributed randomly within blocks and treated statistically as a single, non-contiguous family plot (Lambeth *et al.* 1983).

Four commercial checklots from the North Carolina State University-Industry Cooperative Tree Improvement Program were planted at each test location. The checklots were (1) CC3, North Carolina Piedmont, (2) CC4, Georgia Coastal, (3) CC5, Lower Gulf, and (4) CC7, Georgia-South Carolina Piedmont. At each location, the checklots were rooted cuttings from hedged seedlings. In addition to the rooted cutting checklots, seedling checklots were established at all locations except Claxton and Dublin, Georgia.

Data Analyses

Five-year data were collected on survival, presence of rust galls, height (ft), and d.b.h. (in). Individual tree volume (ft³) at age five was calculated using volume equations derived by Smalley and Bower (1968).

As a result of significant mortality during test establishment at the Claxton and Blakely test sites, we further restricted the dataset by including only those clones with at least one ramet surviving at each of the five locations at age five. Sixty-one clones out of the original pool of 375 met the criterion. In no case did the number of ramets per clone fall below nine. This reduced dataset precluded any detailed genetic analysis (i.e., subdivision of total genetic variance into additive, dominance, or epistatic variance). However, we did compare the different propagule types and estimate total genetic variance and the genotype x environment interaction.

From an earlier study with three of the same tests that are in this study, Paul (1993) learned that unequal error variances were present among the locations. To compensate for these unequal variances, all data in the current study were transformed to a standard normal variate with a mean of 0 and standard deviation of 1. This was accomplished by subtracting the replication mean from

the individual tree value and then dividing by the replication's standard deviation (Snedecor and Cochran 1980a).

For comparing fusiform rust resistance and morphological traits among the propagule types, we only included the three locations where all three propagule types were present. Fusiform rust assessment tallies among the propagule types were compared by using a series of Chi-square tests. The observed rust levels among each checklot propagule type generated the expected frequencies to which the observed frequencies of the superior clones were compared in separate Chi-square tests. We also used this procedure to test the difference between the rooted cuttings of the commercial checklots and their seedling counterparts. Simple t-tests were performed to test for a significant difference between the three propagule types with regard to height, d.b.h., and volume.

In the genetic analysis, the checklot propagules were excluded. Given some imbalance due to mortality, a least-squares analysis was used to calculate the sums of squares. The form of the analysis of variance is presented in Table 3. Variance components were calculated by equating the mean squares with the expected mean squares. The actual coefficients of the variance components were adjusted to compensate for some missing plots (Hartley 1967; Goodnight and Speed 1978). Standard errors for the variance components were calculated according to Becker (1984). All sources of variation were considered to be random.

The estimates of the variance components can be used to help improve the design of future field trials. Because the clone mean is the basis of selection, the error variance of a clone mean is of particular interest; it reflects the precision with which the means are estimated (Snedecor and Cochran 1980b; Foster *et al.* 1984). Also, the error variance of a clone mean, $V_{\bar{x}}$, is used to calculate broad-sense heritability ($H^2_{\bar{x}}$).

The theoretical error variance of a clone mean is

$$V_{\bar{x}} = \frac{\sigma^2_{LC}}{l} + \frac{\sigma^2}{nl}$$

and its broad-sense heritability is

$$H^2_{\bar{x}} = \frac{\sigma^2_C}{\sigma^2_C + V_{\bar{x}}}$$

where $V_{\bar{x}}$ = error variance of a clone mean

$H^2_{\bar{x}}$ = broad-sense heritability, clone-mean basis

σ^2 = error variance component

σ^2_C = clonal variance component

σ^2_{LC} = location x clonal variance component

l = number of locations, 4.7 (adjusted)

n = number of replications per location, 1.7 (adjusted)

To examine the influence of experimental design on results in future experiments, the variance components for height were assumed to be stable while the coefficients, n and l were varied. This is similar to the technique used by Schutz and Bernard (1967) to examine the sensitivity of the standard error of soybean strains to changing allocations of replications, locations and years.

Results and Discussion

Propagule type comparisons. Rust infection levels for the commercial checklots ranged from 5 percent at Dublin to 40 percent at Blakely. Only the test at Blakely met our original expectations of high infection levels. However, at each location, the rooted cuttings of the superior clones, on average, had lower infection levels.

Chi-square analyses revealed that the superior clone rooted cuttings were in fact more resistant to fusiform rust than either their rooted cutting checklot counterparts or seedlings (Table 1). There was also a significant difference in assessment levels between the two propagule types of the commercial checklots. The rooted cuttings had lower levels of rust compared to the seedlings.

Table 1. Comparison of fusiform rust infection levels between the rooted cuttings of superior clones (RC-SC), rooted cuttings of commercial checklots(RC-CC), and seedlings of commercial checklots (S-CC) for three test sites.

Propagule type ^a	Gall type (%)			
	None	Branch	Stem	Branch+Stem
RC-SC	97	2	0	1
RC-CC	80	14	2	4
S-CC	76	18	1	5

^aChi-square analysis reveals a significant difference between RC,SC and RC,CC ($\text{Pr } X^2 > 102.4 = 0.0001$); between RC,SC and S,CC ($\text{Pr } X^2 > 115.8 = 0.0001$); and between RC,CC and S,CC ($\text{Pr } X^2 > 7.38 = 0.0083$).

Regardless of the morphological trait, the superior clone rooted cuttings performed significantly better than either the commercial check rooted cuttings or their seedling counterparts (Table 2). Due to the apparently rapid maturation of loblolly pine (Greenwood 1984; Foster *et al.* 1987) and the relative difficulty of rooting southern pine cuttings, concern had been voiced that the resultant rooted cuttings would exhibit inferior growth patterns. The take-home message here is that rooted cutting propagation and superior clone selection works in loblolly pine.

Table 2. Overall means for height (HT), d.b.h. (DBH), and volume (VOL) at age five for rooted cuttings of superior clones (RC-SC), seedlings of commercial checklots (S-CC), and rooted cuttings of commercial checklots (RC-CC) for three test sites. For a given trait, means with the same superscript letter are not significantly different from each other.

Propagule type	Trait		
	HT (ft.)	DBH (in.)	VOL (cu.ft.)
RC-SC	18.3 ^a	3.8 ^a	0.81 ^a
RC-CC	17.8 ^b	3.5 ^b	0.43 ^b
S-CC	17.9 ^b	3.6 ^b	0.53 ^b

Propagules of similar genetic background can serve as a useful check of the vegetative propagation system. Our results indicated that there was no statistical difference between the

propagation of loblolly pine via rooted cuttings and that via seedlings (Table 2). In other studies, Foster *et al.* (1987) and Foster (1988) have demonstrated that the growth habit for rooted cuttings of loblolly pine is quite comparable to that of seedlings when the cuttings are initially collected from seedlings less than 18 months of age.

Genetic analysis. The results from the genetic analysis revealed that the data transformation successfully reduced location and replication effects (Table 3). This allowed us to focus on the clone and location x clone components of the model.

Table 3. Analysis of variance for the standardized variables height (HT), d.b.h. (DBH5A), and volume (VOL5A) at age five for 61 superior clones of loblolly pine planted in five locations.

Source of Variation	df	Expected Mean Squares	Pr>F		
			HT5A	DBH5A	VOL5A
Location (L)	4	$\sigma^2 + 1.64\sigma^2_{LC} + 100.27\sigma^2_L$	0.9921	0.9828	0.9781
Clone (C)	60	$\sigma^2 + 1.64\sigma^2_{LC} + 8.23\sigma^2_C$	0.0001	0.0001	0.0001
LxC	240	$\sigma^2 + 1.74\sigma^2_{LC}$	0.0497	0.0252	0.0103
Error	234	σ^2			

σ^2_L = variance among locations; σ^2_C = variance among clones; σ^2_{LC} = variance due to interaction of locations and clones; σ^2 = error variance; Pr>F = the probability of obtaining an F-value larger than the one observed.

There were highly significant clone effects for the morphological traits evaluated (Table 3). This source of variation accounted for 14 to 23 percent of the total variation (Table 4). Selecting clones from the upper end of this normal distribution captures both the additive and non-additive genetic variance. This strategy yields a genetic gain greater than that of seedling propagation in which only a portion of the additive variance is exploited. Forest geneticists at N. C. State University estimate that full exploitation of the total genetic potential could increase gain by 4.8 and 9.5 percent for height and volume, respectively, over the expected gains from unrogued second generation seed orchards (Dr. Bob Weir, personal communication).

However, there was also a significant location x clone interaction effect observed for each trait. This source of variation accounted for 9 to 15 percent of the total variation (Table 4). The varying densities among the test plantings, as a result of differing mortality levels, may be one reason that this interaction was so significant, particularly with respect to the density-sensitive traits of d.b.h. and volume. For example, the Claxton test site had the highest mortality level (68%). When that location was removed from the analysis, the P-value of the interaction effect was 0.0498, 0.0713, and 0.0665 for height, d.b.h., and volume, respectively. Survival among the test sites ranged from 68% to 98%.

Another possible explanation for this interaction could be the confounding of ontogenetic age of the ortets with location. Loblolly pine does mature quite rapidly, with discernible expression of some maturation as early as age four years (Greenwood 1984). Repeated hedging appears to delay maturation in other pine species (Libby *et al.* 1972; Libby and Hood 1976), and loblolly is thought to behave in similar fashion. Since the hedges in the current study had been hedged repeatedly, beginning at six months of age, we assumed that the maturation effect would be negligible from 18 months (age of cuttings in the 1987 tests) to 30 months (age of cuttings in the 1988 tests). However, this may not be the case.

Table 4. Variance components (\pm SE), their percentage of total variation, and broad-sense heritability estimates (H^2_X) for standardized height (HT5A), d.b.h. (DBH5A), and volume (VOL5A) at age five for 61 superior clones of loblolly pine planted in five locations.

Source of Variation	Trait					
	HT5A		DBH5A		VOL5A	
	Variance component \pm SE	% Total Variation	Variance component \pm SE	% Total Variation	Variance component \pm SE	% Total Variation
Location (L)	0	0	0	0	0	0
Clone (C)	0.22 \pm 0.05	22.7	0.13 \pm 0.04	13.5	0.16 \pm 0.05	16.5
LxC	0.09 \pm 0.07	9.3	0.12 \pm 0.06	12.5	0.14 \pm 0.06	14.5
Error	0.66 \pm 0.06	68.0	0.71 \pm 0.07	74.0	0.67 \pm 0.06	69.0
Total	0.97		0.96		0.97	
H^2_X ¹	0.68		0.54		0.59	

¹The adjusted coefficients for n and l used to calculate broad-sense heritability were 1.7 and 4.7, respectively.

With respect to height, the interaction component is 42% of the clonal component of variance (Table 4). Shelbourne (1972) suggested that, as a rule of thumb, if the interaction component reaches 50% or more of the entry (clonal) component of variance, then the effects of the location x clone interaction are likely to cause problems for selection and testing. If the location x clone interaction is real and not the result of confounding with ontogenetic age, then one may have to consider establishing clonal testing zones in order to maximize gain from clonal selection.

Broad-sense heritability on a clone-mean basis was 0.68, 0.54, and 0.59 for height, d.b.h., and volume, respectively (Table 4). Unless clones are to be selected on the performance of a single rooted cutting or a single plot, the appropriate heritability estimate should be based on clone means. These heritabilities are lower than previously reported for this same population of clones. Paul (1993) estimated H^2_X for height, d.b.h., and volume to be 0.90, 0.96, and 0.94, respectively. One possible explanation could be that there are more locations in this analysis compared to Paul's study.

Experimental design and the allocation of resources. The goal of a design for estimating clone means is to minimize the error variance of a clone mean ($V_{\bar{x}}$) for a given cost (Russell and Libby 1986). With respect to height growth, the greatest decrease in $V_{\bar{x}}$ occurs as the number of locations increases from two to four (Figure 1). Additional locations do not greatly reduce the error variance of a clone mean. Four locations should be adequate to sample divergent rust strains and promote selection for generally resistant clones. Given the constraint of four locations, we can decrease $V_{\bar{x}}$ further if we increase the number of blocks from two to three.

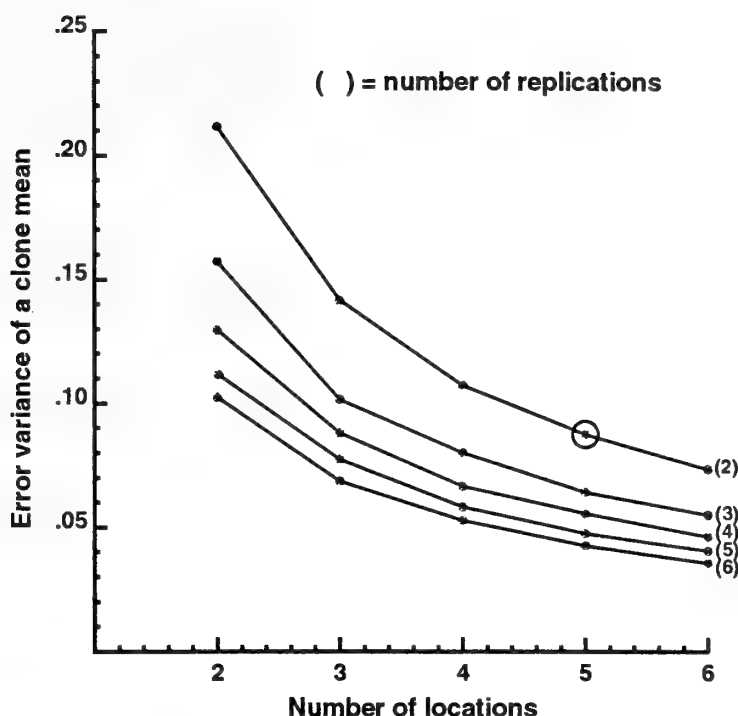


Figure 1. Error variance of a clone mean for height for different numbers of locations and replications per location. The circled data point indicates the experimental design of the current study.

While the current experimental design is good, the modified design would allow us to reduce both $V_{\bar{x}}$ and the cost of test establishment and maintenance by consolidating our efforts on fewer sites. The added cost of increasing the number of blocks at a given location from two to three should be offset by the reduction in the number of locations. It will also allow us to conserve a valuable resource, the industrial cooperator. Without them, a lot of these field trials would never be planted.

CONCLUDING REMARKS

Loblolly pine cuttings can be taken in March, June, and October of a given year. Juvenility, hedge health, and consistent environmental parameters are all critical to rooting success.

Five-year data reveal no significant difference between seedling and rooted-cutting propagules of similar genetic background, when the hedged donor plant is less than 30 months of age. Rooted cuttings of select clones perform better, on average, than the commercial checklots, both in terms of fusiform rust resistance and morphological traits.

There is a strong clonal effect for the morphological traits evaluated. The possible $G \times E$ interaction suggests that one may have to establish clonal testing zones in order to maximize gain from clonal selection. Sensitivity analysis on the error variance of the clone mean suggests that in future field trials, one could reallocate their resources to four test sites and three replications per site.

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**LOBLOLLY AND SLASH PINE ROOTED CUTTING RESEARCH
AT N.C. STATE UNIVERSITY**

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Abstract. -- Through the efforts of several N.C. State University scientists and with support of nine forest product companies, a loblolly and slash pine rooted cutting research project was initiated in January of 1992. Focus of the research is on: (1) genetic selection (culling) for rooting ability, (2) physiological process research on the fundamental mechanisms for adventitious root initiation, (3) stock plant or hedge physiology and culture with emphasis on carbohydrate / nitrogen ratios in the stem cuttings produced, (4) rooting environment research, and (5) investigation of auxin treatments for stimulating rooting and their effect on root system quality.

Open-pollinated loblolly families (48) have been screened using a hypocotyl rooting system to find hard and easy to root families that are now being used in additional physiological mechanism studies. Open-pollinated slash pine families have been rooted using the hypocotyl system and the family mean correlation between the number of roots per hypocotyl and volume or rust are .055 (ns) and .148 (ns) respectively. Early rooting experiments with IBA and P-ITB (an aryl ester of IBA) show no strong response to auxin with cuttings from 1-year old hedges, yet family rooting differences were substantial.

Keywords: Loblolly pine, slash pine, rooted cuttings,

INTRODUCTION

Rooted cutting technology leading to clonal forestry offers enormous potential for increasing forest productivity and value. In many parts of the world, intensively managed forest plantations established with rooted cuttings of outstanding genotypes are producing high value / low cost wood for an increasingly competitive global forest products industry. Despite many years of research and development work, a reliable, cost-effective, rooted cutting system for difficult to root loblolly and slash pines is not available. We have organized, with the support of nine forest industries, a research initiative aimed at studying the

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fundamental processes of adventitious root initiation and development with complementary work aimed at developing a useful rooting technology for these species.

RESEARCH FOCUS and PROJECT ORGANIZATION

The project is organized with five primary areas of research emphasis. All five areas will contribute to an increased understanding of adventitious root initiation and development, and/or work toward refinement of a useful rooted cutting technology that will allow the full potential of clonal forestry to be realized for loblolly and slash pines. Significant interaction and interconnection exists between and among research areas. However, for purposes of description, the five primary areas of research are described separately below.

1. **Genetic Selection (culling) for rooting ability** is being evaluated for its potential to enhance rootability and the subsequent impact on genetic gain for growth and rust.
2. **Molecular biology** studies are concentrating on understanding the role of gene expression in the physiology and developmental process of adventitious root initiation. The work includes studies of polar auxin transport, auxin binding proteins and research on genetic control of maturation and the accompanying loss of rooting.
3. **Stock plant physiology** and culture with initial emphasis on managing the carbohydrate/nitrogen ratio in hedges and the stem cuttings produced is being evaluated.
4. **Rooting environment research** is focusing on the evaluation of a containerized "plug-nursery" system. This work will compare various rooting media, containers for rooting, seasons of the year for harvesting and sticking cuttings, as well as greenhouse versus shade house environments for rooting.
5. **Root system quality** developed on rooted propagules is being studied in relation to varying auxin treatments used to stimulate root initiation.

An N.C. State University research team comprised of physiology, molecular biology, genetics, and horticulture specialists has been assembled to conduct this research. Dr. Michael Greenwood, Ruth Hutchins Professor of Tree Physiology, University of Maine, served as lead visiting scientist for the project in year one. In year two physiologist / molecular biologist, Dr. Barry Goldfarb, Assistant Professor and Rooted Cutting Project Lead Scientist, N.C. State University joined the project. Work on rooting environment and propagule quality research is being done by Dr. Frank Blazich, stock plant nutrition studies by Dr. Leslie Henry, and genetic selection work and overall project coordination by Dr. Robert Weir. The research project duration is for four years with a decision to continue contingent on progress and additional research needs.

RESEARCH PROGRESS AND PLANS

Work has been underway on the rooted cutting research project for 18 months and to some extent we are still in the "getting started phase" of activity. However some results have been obtained and are reported below along with a description of study plans and additional work in progress.

Genetic Selection (Culling) for Rooting

Screening and selection of families for rooting ability is an obvious way to enhance the efficiency of a rooted cutting system. Genetic variation for rooting is substantial and the genetic control of rooting in loblolly pine appears to be strong. Broad sense heritability for rooting percent in loblolly pine was estimated from a small group of families (9) to range from .61 to .86 (Anderson et. al., in preparation). In a larger study involving 54 families, but with a small number of donor ortets per family, Foster (1990) reported broad sense heritabilities for rooting percent in the range of .20 to .40.

Selecting for rooting percentage may constrain the improvement possible for growth and productivity traits. Some hope can be derived, however, from the results of Foster (1985). In western hemlock, he found a positive genetic correlation (+.37) between rooting percent and subsequent growth of the rooted cuttings. While the literature abounds with evidence of family differences for rooting ability, little has been reported on the genetic gain consequences of applying this selection / culling to a population of fast growing families. This can be conceptually thought of as an independent culling problem. Select many outstanding families for growth superiority and then propagate only those families or individuals within family that have acceptable rooting percentages (75 + percent?). The question then becomes: what proportion of the good families must be discarded for poor rooting and how does the commensurate reduction in selection intensity impact growth and/or rust improvement?

Initial research efforts in this area are focused on establishing these relationships for slash pine. A reasonably good protocol (resulting in 70+ percent rooting) for rooting hedged slash pine currently exists. Seeds from 50 slash pine half-sib families from the University of Florida Cooperative Forest Genetics Research Program have been obtained. These families represent a wide range of good and poor volume and rust resistance performance. Cutting hedges, derived from 20 seedlings per family, have been started for each of the 50 families. A screening trial for rootability will be conducted to establish the relationship between culling for rooting percentage and the subsequent impact on gain for growth and/or rust resistance. Similar work will be done for loblolly pine, once physiology studies have provided information to improve the rooting protocol.

While we are developing hedges of the 50 slash pine families for rooting trials using stem cuttings, we have screened these same families for rooting ability using an experimental hypocotyl rooting system developed for loblolly pine by Michael Greenwood. In this experimental

system, newly germinated slash pine seedlings (21 days from sowing) have their roots removed by severing the stem 2.5 cm below the cotyledons. Each hypocotyl cutting is placed in a hole drilled in a styrofoam sheet which is floated on a solution of 10 micro-molar IBA (Indole-3-butyric acid) and distilled water contained in an opaque plastic tray. Approximately 1 cm of hypocotyl stem is constantly bathed in the IBA solution. The cuttings are placed in a growth chamber and maintained at 80° F day, 68° F night, with a 16h photoperiod with 1000 to 3000 foot candles of incandescent light.

With IBA treatment, virtually all hypocotyl cuttings initiated new roots after 20 days. The most useful rooting response was the number of roots per hypocotyl at 15 days after sticking, as this trait displayed substantial family variation and, as discussed later for loblolly, correlated to some degree with cuttings from one to three year-old hedges. The family means for number of roots per hypocotyl ranged from a low of 4.1 to a high of 24.0. Narrow sense heritabilities for root number were .44 on an individual tree basis and .63 calculated on a family mean basis. Culling the lowest 50% of the families increased the mean number of roots per cutting from 12.4 to 16.0. The family mean correlation between root number and volume was .055 ns, and between root number and rust resistance was .148 ns. These results indicate that screening slash pine families for rooting ability could enhance the efficiency of a rooted cutting system without negatively impacting gains in volume and rust. These results should be viewed with caution, however, because the strength of the correlation between hypocotyl rooting and rooting of cuttings from hedges is still uncertain.

Molecular Genetics and Cell Biology of Root Initiation

Michael Greenwood developed a system for studying the importance of auxin in the rooting process using hypocotyl cuttings from loblolly pine seedlings placed in a solution containing 10 micro-molar IBA (Greenwood, 1992). He demonstrated that auxin treatment is necessary for root formation in these cuttings and when applied yields almost 100% rooted cuttings. Phytotropins (auxin transport inhibitors) applied at the same time as the IBA, inhibited root formation implicating auxin transport and possibly auxin binding as having critical roles in root initiation. A logical next step will be to examine a class of proteins known as auxin binding proteins. These have been hypothesized to be auxin receptors (Jones and Prasad, 1992). It will be informative to learn if these putative receptors differ in good and poor rooting genotypes, different tissues of the seedling, and stem tissues from trees of varying ages.

A major research focus is on gene expression during rooting and how expression of rooting genes changes during tree maturation. A hypocotyl cutting rooting system similar to the one described above for eastern white pine has been developed (Goldfarb et al, 1992) and is being adapted to loblolly pine. It differs from the previous system in that the hypocotyl cuttings are given a high-concentration (300 mg/L or 1.6 mM) pulse with 1-naphthaleneacetic acid (NAA) for 5 minutes and then transferred to water or moist sand for the root initiation period. This

system is ideal for studying changes in gene expression during rooting, because a large number of roots are formed on each cutting (~20 for loblolly).

Gene expression will be studied by identifying genes expressed uniquely in root-forming pine cuttings using cDNA cloning and differential or subtractive hybridization. In addition to pine rooting genes identified in this manner, we can also use gene probes from other plants that may be involved in the rooting process. Candidate probes include a gene from tobacco that is expressed in root meristems (Conkling et al, 1990; Yamamoto et al, 1991), a cell-wall glycoprotein from English ivy whose expression is negatively correlated with adventitious root initiation (Wu et al, 1993), genes stimulated by auxin treatment in soybean hypocotyls (Li et al, 1991), and genes controlling transcriptional regulation during flower development in Arabidopsis (Coen and Meyerowitz ref) and leaf development in maize (Vollbrecht et al, 1991).

We are also collaborating with the Forest Biotechnology group at N.C. State to use RAPD mapping to analyze which portions of the genome contribute to variation in rooting success (quantitative trait loci or QTL analysis). One thousand seedlings from a single open-pollinated family have been screened for rootability in the IBA hypocotyl system and the DNA from the female gametophytes of those seedlings is being analyzed.

We intend that these approaches will provide us with a better understanding of the fundamental mechanisms of root initiation and ultimately with the information needed to design better rooted cutting systems.

Stock Plant or Hedge Physiology and Culture

Evidence reported in the literature suggests that the environmental history and the resulting physiology of a stock plant or hedge strongly influences subsequent rooting of stem cuttings. Two measures of physiological status which are strongly influenced by stock plant environment are carbohydrate and nitrogen contents (Moe and Anderson 1988). Changes in the relative amounts of either carbohydrates or nitrogen within cuttings have been shown to influence rootability (Haissig 1986) .

Although a regulatory role for carbohydrates in the rooting process has not been firmly established, it is understood that total non-structural carbohydrate (TNC = soluble sugars and starch) content within a cutting may influence rooting by establishing the amount of energy reserves and carbon skeletons available to support root initiation and growth (Haissig 1986; Veierskov 1988). Carbohydrate level may also influence mobilization, including loading and/or transport, of auxin from the leaves into the roots (Jarvis 1986). However, carbohydrate status alone does not necessarily determine rootability. Depending on the species, a high concentration of TNC at the time of severance may show a positive, negative or zero correlation with subsequent rooting (Veierskov 1988).

Nitrogen status of the stock plant may also influence rootability of cuttings (Moe and Andersen 1988; Haissig 1986). Nitrogen deficiency (not stress) within the stock plant generally promotes root formation of cuttings, possibly via its influence on carbohydrate metabolism (Haissig 1986). When nitrogen is deficient, further metabolism of the carbohydrates produced by the stock plant into organic compounds is restricted, and sink demand for carbohydrates to support shoot growth declines. Under these conditions, excess carbohydrates can be translocated as sucrose and stored within the stem tissues as a combination of sucrose and starch (Veierskov 1988). The greater levels of carbohydrates within the stem tissues would then be available to support root formation on cuttings. This indicates that it is not nitrogen status *per se* that influences subsequent rooting. It is rather the influence of nitrogen status on the amount and form of carbohydrate present within the stock plant prior to severance that influences root formation on cuttings. Once severed, nitrogen status within the cutting determines whether the available carbohydrate will be directed toward further shoot production or toward root formation: a high nitrogen status relative to available carbohydrate results in a tendency for stored carbohydrate and current photosynthate to be directed into shoot production.

In addition to the influence of environmental effects, stock plant physiology is influenced by the genetics of the individual plant. Correlations between carbohydrate / nitrogen ratio and rootability have been shown to have a genetic component (Struve 1981). Previous research with *Pinus rigida* has indicated that differences in rootability of clones may be related to differences in their carbohydrate / nitrogen ratios (Hyun and Hong 1968): a clone which rooted easily had a higher carbohydrate / nitrogen ratio throughout the year than did the clone which was difficult to root. Differences in the carbohydrate / nitrogen ratio were attributed to differences in the nitrogen content, with the easy-to-root clone having a lower nitrogen content than the difficult-to-root clone. The greatest differences in carbohydrate / nitrogen ratios between clones coincided with the time of year when rooting differences were at a maximum.

To determine the effects of stock plant nutrition on rooting of cuttings, hedge plants with differing carbohydrate / nitrogen status are being established by growing plants under a range of nitrogen availabilities (5, 10, 15, 20, and 50 ppm N supplied as NH_4NO_3 ; all other nutrients will be supplied at optimal levels). A sixth (control) treatment is also included that consists of a standard Osmocote fertilization regime. These treatments include an upper level of N which gives optimal growth of loblolly pine seedlings and a lower limit which causes a shift in carbohydrate partitioning from shoot to root growth. These treatments and the intermediate levels of N bracket the level of N (20 ppm) which has been shown to produce maximal rooting response in cuttings from red cedar stock plants (Henry et al. 1991). Hedges of two full-sib families of loblolly pine that have previously shown to have consistently high rootability (>50%) and two full-sib families that have shown consistently low rootability (<10%) are being used in the study.

A factorial design of families and nitrogen treatments is being utilized. Each family / treatment combination is represented by four hedges in each of four randomized complete blocks. The complete study is comprised of 384 hedges. Hedge plants for the five nitrogen treatments are being grown in a sand / perlite medium, while the seedlings in the Osmocote control are growing in a medium of 2 parts peat, 2 parts vermiculite, and 1 part perlite. The first hedging and rooting experiments will be conducted in January 1994 with a second hedging to follow in May 1994.

The total non-structural carbohydrates (sucrose, reducing sugars, and starch) and total nitrogen (soluble and insoluble fractions) will be determined from analysis of the cuttings. These analyses will provide baseline information regarding the physiological status of the hedge plants at the time the hardened cuttings were removed. Rootability of cuttings from each nitrogen treatment will be determined under an "optimal" rooting environment in the greenhouse. Percent rootability will be compared between nitrogen treatments within a family. Correlations between rootability and carbohydrate and/or nitrogen status (including concentrations of each form of carbohydrate and/or nitrogen present) will be established for each treatment within a family. Since nitrogen availability will affect vegetative growth of the hedges, the effects of nitrogen treatment on the number of orthotropic shoots produced by hedged plants will also be determined.

Rooting Environment Research

Considerable research activity has been directed in recent years toward manipulation of the rooting environment. Some success has been achieved recently with trials that mimic the New Zealand approach to rooting radiata pine in an outdoor nursery bed environment (Frampton and Hodges 1989). Yet for every success, several failures have occurred. While the New Zealand system offers some promise in the southern U.S., the demands for excessively well drained rooting beds, high volumes of water applied as a high frequency fine mist, and the need for protection from mist disrupting and desiccating winds, may limit the usefulness and cost effectiveness of this rooting system in standard nursery beds. An alternative rooting system is being evaluated.

We have begun work on a containerized greenhouse / shadehouse "plug-nursery" system for producing rooted cuttings. This approach provides the following advantages:

1. Utilization of existing automated equipment developed for plug-one seedling production in the Pacific Northwest.
2. Improved control of environmental variables during the rooting period, especially temperature control.
3. The production of multiple rooted cutting crops per year thus providing cost benefits on facility production rates by increasing the utility of stock plant hedges overtime and by more evenly distributing personnel demands.

In developing a "plug-nursery" research effort we will examine the effect of container size, shadehouse / greenhouse environments, rooting in different seasons of the year, nutrition management, and rooting media. Rooting media effects could prove to be interesting in that the type of root system has a profound effect on lifting and outplanting of the rooted cuttings. Whether a coarse or fibrous root system develops on a cutting is strongly influenced by the rooting medium. For example, cuttings of some species, when rooted in sand, produce long, unbranched, coarse and brittle roots. However, when rooted in a mixture of medias, such as sand and peat moss or perlite and peat moss, the roots that are produced are well-branched, slender and flexible. The latter type is more suitable for digging and outplanting (Hartmann and Kester, 1983). If a standard protocol is to be developed for mass propagation of stem cuttings of pines, research is needed to determine which media will produce rooted cuttings of a quality that will reasonably assure survival and performance following outplanting. The criteria for evaluation will include: rooting percentage, plant quality, transplanting ease, and cost differences.

Quality of Rooted Propagules

During vegetative propagation of various woody species by stem cuttings, treatment of the cuttings with root promoting compounds, most notably auxin(s), is a common practice. Although many species respond positively to auxin treatment, some do not, illustrating that a positive physiological response to auxin treatment is not universal. However, the benefit of auxin treatment is obvious in those species where rooting would not otherwise occur. In addition, four specific advantages associated with auxin treatment have long been recognized:

1. Increasing the percentage of cuttings that form roots.
2. Hastening root initiation.
3. Increasing the number and quality of roots produced per cutting.
4. Increasing the uniformity of rooting.

The first, second and fourth advantages need no explanation. However, the third, regarding the number and quality of roots per cutting, is intriguing because countless studies have noted it as a benefit of auxin treatment. Published reports on the influence of root numbers on establishment and subsequent cutting growth are conflicting yet indicate the possibility of a positive affect (Struve et al., 1984; Struve and McKeand 1990; Wisniewski et al., 1991).

When rooting stem cuttings of various pine species, root number per rooted cutting is usually quite variable within specific treatments regardless of percentage rooting for a treatment. Even though a particular treatment may yield a high rooting percentage, rooting percentage by itself is probably not a good indicator of a treatment because the number of roots on individual cuttings must also be

considered. Although it appears logical that root number per rooting cutting is an important factor to consider when evaluating the relative effectiveness of particular treatments, the extent, as mentioned previously, to which root numbers influence establishment and subsequent cutting growth is unclear, especially for loblolly and slash pines.

To establish the relationships for rooting percent, root number and auxin treatment, a series of initial greenhouse rooting experiments are underway. The first experiment used cuttings from 100, one-year-old seedling hedges grown from four full-sib families. Harvested cuttings of 9 cm length were dipped (basal 1 cm) in auxin solutions, stuck in a raised greenhouse rooting bench, containing a media of 1 part peat and 1 part perlite. The cuttings received intermittent mist 6-8 seconds every five minutes for 13 hours each day to reduce desiccation. The auxin treatments used were 5, 10, 15, 20, and 25 mM indole-3-butyric acid (IBA) and an aryl ester of IBA [phenyl indole-3-thiolobutyrate (P-ITB)], plus a control. The aryl ester of IBA is a relatively new synthetic auxin and in some cases, has been superior to the free acid of the same compound in promoting root initiation (Blazich, 1988; Haissig, 1979; 1983). The experiment consisted of 6 replications of four families and 11 auxin treatments. There were six cuttings in each rep / family / treatment combination for a total of 1584 cuttings.

Overall rooting percentages were good, however, the auxin treatments did not appear to promote rooting, as the percent rooting for the control was always equal or higher than the auxin treatments (Figure 1). The aryl ester of IBA, P-ITB, at low concentrations, was associated with increased root numbers per cutting (Figure 2). Family differences for rooting percent were evident (Figure 3).

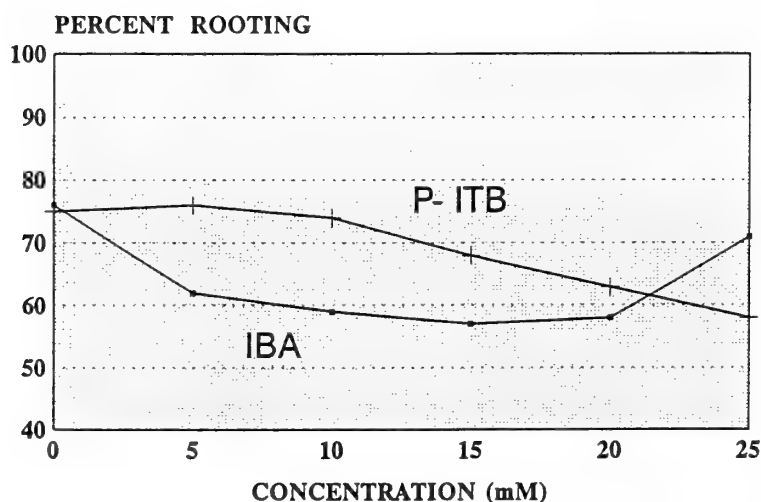


Figure 1. The effect of IBA and P-ITB on rooting percent of loblolly stem cuttings from 1-year-old hedges.

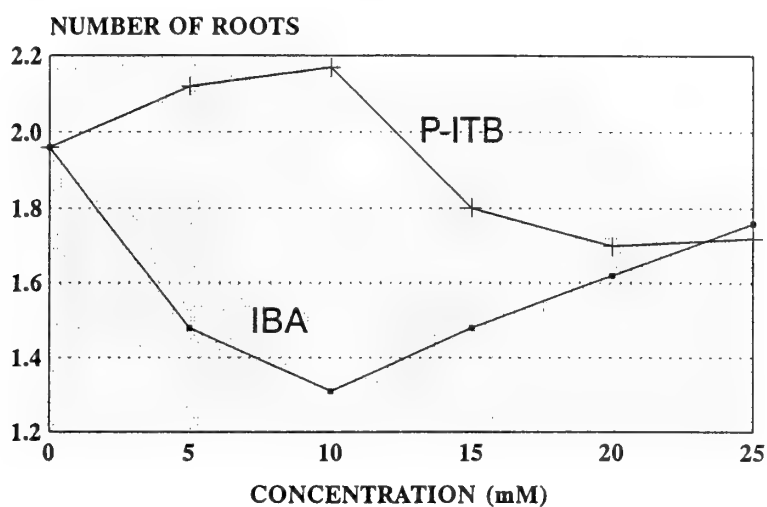


Figure 2. The effect of IBA and P-ITB on the number of roots on loblolly stem cuttings from 1-year-old hedges.

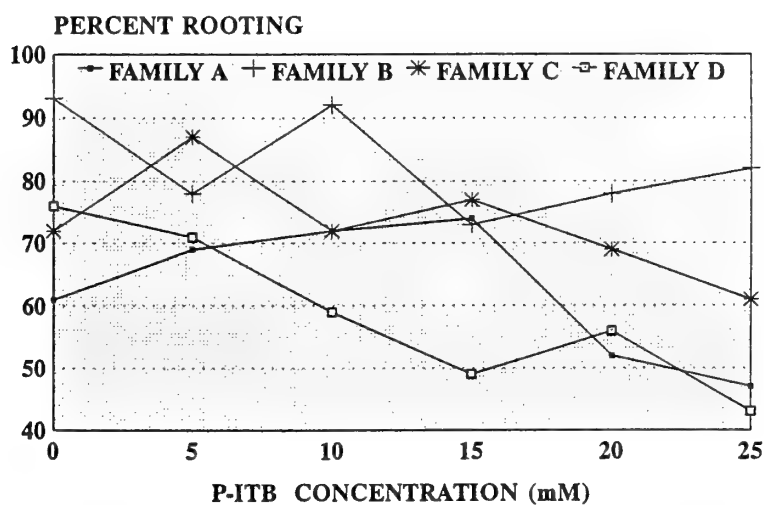


Figure 3. The effect of P-ITB on percent rooting for four families of loblolly stem cuttings from 1-year-old hedges.

Future work in this area will evaluate additional hormone treatments, including Hare's powder, and possibly other synthetic hormone compounds. It may be that as the hedges age and maturation commences, the hormone treatments will provide a greater root initiation stimulus. We also plan to evaluate the season of cutting harvest, especially comparing dormant season (hardwood) cuttings to succulent cuttings harvested in May and August during the growing season. A long term goal would be to develop quality standards for rooted cutting propagules, based on root numbers, or comparable criteria that may prove to be important. Outplantings will be established for subsequent survival and growth comparisons among quality classes.

SUMMARY

A research project focused on improving our basic knowledge concerning adventitious root initiation and development on loblolly and slash pine rooted cuttings has been initiated. The project encompasses a broad range of investigation from the very basic molecular biology studies, including gene expression, to more applied genetic selection and rooting environment studies. Some intriguing early results have been achieved, yet much remains to be done. The ultimate goal is to develop sufficient understanding about the fundamental process of adventitious root initiation and development to allow the development of a practical, cost effective rooted cutting system for loblolly and slash pines that will lead to the implementation of clonal forestry for these two regionally important pine species.

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SIMULATION MODELS FOR BREEDING POPULATION ADVANCEMENT

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Abstract. -- Parameter based simulation modelling was used to look at mating designs and population structure for breeding population advancement. The use of balanced vs unbalanced parental contribution to gain and effective population size was investigated. Non-random unbalanced mating adds to gain but at marked reduction in effective population size. The trade-off between gain, and maintaining genetic diversity as described by effective population size, was best made using balanced mating and truncation selection than the use of unbalanced or assortative mating designs. The effect of subline size indicated smaller sublines provide lower gain but higher overall population diversity.

Keywords: Advanced generation planning, simulation models, genetic gain, effective population size, sublines.

THE SIMULATION MODELS

A Monte Carlo simulation model was designed to investigate features of breeding population advancement (King and Johnson 1993). The details of the model are based on the New Zealand radiata pine improvement program but have in common many of the features of tree improvement programs using recurrent selection for general combining ability (Burdon and Shelbourne 1971, Shelbourne et al. 1989). This includes: a complimentary mating design one for GCA testing and another to create a population for recurrent selection; different testing features including single-tree-plots for the GCA test and family blocks for within-family selection in the recurrent-selection population; assortative or non-random mating in the recurrent-selection crosses; the structuring of the breeding population into replicates or sublines - inbreeding is contained within sublines and outcrossing is assured in the production population by crossing between sublines. The modelling uses a Monte Carlo simulation of the advancement of the breeding population for five generations of recurrent selection for a single trait. The model is parameter based and estimates of genetic and environmental variances were used to produce independent normal distributions of true genotypic values and environmental deviations. Phenotypic values constructed from the sum of these two independent distributions provided the basis for the selection of a simulated breeding population. Mating and the generation of an offspring population were constructed under the assumption that

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genotypic values for the trait derive from a polygenic and purely additive gene effects model. King and Johnson (1993) provides the details of the model and the assumptions that were used. The simulation model was designed to address the last two features noted above particularly the effect on both genetic gain and effective population size of using non-random mating features and different population sub-structuring.

Model for Breeding Population Advancement

Lindgren (1986) proposed promoting some of the features of assortative mating by the use of imbalanced family contribution for breeding populations. This comprises having both more representatives of good families and more cross combinations with better genotypes. Increasing mating frequency provides another level of selection over initial truncation selection. Comparisons between mating designs for recurrent selection populations should include not just genetic gain (after all increasing family selection will produce higher gain) but also retaining genetic diversity within the framework of a fixed population resource. Effective family number involving the reciprocal of the sum of squares of each parent's proportional contribution to the breeding population offers a simple and effective way of measuring genetic diversity (Robertson 1961, Kang and Namkoong 1988).

The comparisons provided in these simulations are all made with a breeding population size of 60 parents and an offspring population of 6000. The details are provided in King and Johnson (1993) and include: double pair cross design random mated; imbalanced mating design with top third out of the 60 index ranked parents crossed three times, mid third crossed twice and the bottom third crossed once; double pair cross design with an additional group of crosses involving the top 6 parents in a half diallel; and the 60 parents crossed 8 times each randomly.

In brief, the results showed that imbalanced mating produced significantly more gain (about 10%) than random balanced mating, however having more cross combinations produced higher levels of gain (about 20%) (King and Johnson 1993). Heritabilities are not high enough that assortative mating can accurately match the best genotypes. Increasing mating frequency offers a better opportunity that the best genotypes will be matched. Effective population size was more quickly reduced with imbalanced mating than with random mating for a given level of gain at lower selection intensities. At high selection intensities this difference was less noticeable. Gain and diversity are best managed in the breeding population through the selection process. A combination of high mating frequency and assortative mating is best when selection objectives also incorporate the production population where genetic diversity is less of an issue.

MODELLING POPULATION SUBSTRUCTURE

Background

Wright (1922) concluded that improvement with artificial selection would be more rapid if a population were subdivided into small lines. Wright stressed that this conclusion was dependent on selection being for epistatic combinations. Reviews of many studies in artificial selection and animal improvement shows no advantage to population subdivisions when traits are under additive gene action (Barker 1989, Lopez-Fanjul 1989). Population substructuring is not expected to be as efficient as managing larger populations except: 1-selection across sublines may provide some rapid gain as small sublines are more quickly fixed for favorable alleles - this is temporary as larger sublines will soon overtake the smaller sublines (Madalena and Hill 1972); and 2-small sublines are expected to be more efficient for the elimination of deleterious recessives (Madalena and Hill 1972). Another potential advantage, however, to having population sub structure is to help in maintaining genetic diversity. Small sublines provides a method of maintaining many unrelated sub structures thus providing for a wider base. This is expected to occur at a loss to overall gain with the ability to use higher selection intensities in larger sublines.

The model here used a breeding population of 100 parents. The treatments comprised: the population maintained as one undivided breeding population; 5 sublines of 20 parents each; and 10 sublines of 10 parents each. The mating design comprised a random double pair cross and in addition the top selections were crossed in a half-diallel to produce elite crosses (e.g. EN; King and Johnson 1993). Each population treatment provided 100 crosses as random double pair crosses and 30 crosses in elite crosses. 100 individuals were created for each full-sib family for an offspring population of 13,000. Selections using combined index selection (King and Johnson 1993) were made in the offspring to choose 100 parents for the next generation of breeding. A restriction of a maximum of 4 individuals per full-sib family was made. Over 100 independent runs for two cycles of recurrent selection were made for these three treatments.

Results of Simulation Runs

The results of the simulation runs are summarized in Table 1. Gains are shown as percentage gain over the previous generation mean averaged over two cycles of recurrent selection. Effective population size calculated as per King and Johnson (1993) refers to the weighted parental contribution in the second cycle of recurrent selection from the 100 parents of the breeding population. There are higher levels of genetic gain in the undivided breeding population, nearly 30% more per generational gain than the smallest subline size but the effective population size is less than half of that present in the smallest subline size.

Table 1. Per Generation Gain and Effective Population Size for Differing Subline Sizes.

Treatment	Gain	Ne
1 x 100 parents	13.5%	17
5 x 20 parents	11.5%	30
10 x 10 parents	10.5%	38

Using small subline sizes thus presents a cautious way that genetic diversity can be maintained in the breeding population. These results should be treated as preliminary, many questions beg answering. For instance, can restrictions be used in the selection process to maintain higher effective population size in the larger subline and how will gain be affected this way? Is this cautious approach the best way of managing for genetic diversity? Small sublines would be expected to diverge with fixation of different alleles due to drift. Directional selection that allow divergence because of selection for different traits and objectives is another way that genetic variability can not only be maintained but enhanced. Selection in this way for multiple populations is different than the division of the breeding population into replicate populations or sublines. Sublines are not expected to have different selection objectives and in tree improvement are meant primarily to control inbreeding in the production population by containing it within sublines (Lowe and van Buijtenen 1981). Sublines should also be distinguished from breeding groups such as disconnected diallels that are established for manageability but can be selected across groups. The subline unit as a replicate of the breeding population is meant to contain useful genetic variation within lines (Lowe and van Buijtenen 1981). Multiple populations or varietal lines can be contained within sublines, although this would be difficult if unfavorable correlations exist. The use of multiple populations in this way may be a more active way of maintaining and enhancing the genetic resources of our tree species than the conservative approach of using small sublines (Namkoong 1984). Large sublines can provide more flexibility to provide selections for multiple breeding objectives.

CONCLUSIONS

Monte Carlo simulations offers a valuable tool in planning and quantifying strategies for advanced generation breeding programs. In a selective breeding program continued improvement and the management of diversity is best made in the selection process. Mating designs and population structure should be used to enhance the selection opportunities to meet these goals.

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APPLICATION OF GENETIC MARKERS TO TREE BREEDING

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Rapid advances have occurred concerning DNA markers in forest genetics:

Two years ago, in June 1991, a workshop was organized by G.A. Tuskan and C.G. Williams, at Gatlinburg, Tennessee, on the potential use of genetic markers in tree breeding (Tuskan 1992). Few if any of the attendees would have predicted the rapid advances that have taken place in theory and methodology that can now be applied to genomic analysis of forest trees. The purpose of this paper is to reexamine some of the issues raised at the Gatlinburg meeting in view of this new technology and new theory.

High levels of genetic polymorphism in many forest tree species and the use of PCR based markers make possible moderate density genomic maps for individual trees. Map construction can be completed in weeks instead of years because PCR-based marker analysis has been automated (Nelson et al 1992a). The ability to map virtually any sexually mature tree (with sufficient heterozygosity) provides a basis for the reevaluation of the potential applications of genetic markers for tree breeding, as well as the potential application to studies of genetic diversity and selection in natural stands of forest trees.

Why were we skeptical two years ago? Widespread doubts were expressed about the utility of DNA markers because of both theoretical and technical limitations. Markers were considered to have limitations even in agronomic crops where genetic analysis was more advanced. Forest trees had high levels of linkage equilibrium, precluding predictable associations of markers and QTLs. No QTLs had been demonstrated in any forest tree. Finally, the technology required for mapping was expensive and relatively inaccessible to tree breeders. Strauss et al (1992) stated, in their contribution to the workshop, "we expect that the near term usefulness in most operational tree breeding programs will be limited". Furthermore, variation of quantitative trait effects depend upon genetic background, genotype by environment interaction, and variation over generations. Nonetheless, it was argued that marker aided selection had potential application for weakly heritable traits (Lande and Thompson 1990) where markers can "explain much of the additive variance within families".

Impact of PCR-based markers: Two factors have made it relatively easy to generate genetic maps of individual trees: the technology of PCR based markers, and the high levels of heterozygosity in most forest tree populations.

The first major obstacle to genomic mapping and marker analysis in forest trees was the problem of linkage equilibrium. In the absence of extended pedigrees, inbred lines or specific cultivars or other situations where linkage disequilibrium could be readily established, it was unlikely that specific associations of markers and important traits could be made. Several approaches have been taken to establish linkage disequilibrium for genetic mapping in forest trees.

Neale and coworkers (Neale and Williams 1991) have used a three generation pedigree of loblolly pine directed to the mapping and dissection of quantitative traits such as wood density (Williams and Neale 1992). Bradshaw has analyzed markers in an interspecies cross of *Populus trichocarpa* x *deltoides* (clone H11) (Bradshaw and Stettler 1992). Both Bradshaw and Neale used restriction fragment polymorphisms (RFLPs) as DNA based markers in pioneering studies of genomic mapping in trees. These markers provided a large number of polymorphic genetically neutral markers that made the earliest maps possible. In spite of these advances, most forest geneticists viewed RFLP marker analysis as laborious and limiting. Furthermore, extended pedigrees of adequate size were rare.

The high levels of heterozygosity in forest trees are an advantage for application of molecular markers. The third approach to achieve linkage disequilibrium is the use of individual trees for genetic analysis. This approach was pioneered years ago using isozymes (Conkle 1981) but was limited by the small number of enzymes for which assays were available. Isozyme markers have proved useful for the estimation of genetic variation, levels of heterozygosity, gene flow, genetic mapping and genetic relatedness. In contrast with agronomic crops, high levels of genetic polymorphism and heterozygosity were turned to advantage for genetic analysis of forest trees (e.g. Conkle, 1981; Wheeler et al 1983; Hamrick and Godt 1990; Millar et al 1988).

The advent of PCR based markers: In 1990, two research groups, Welsh and McClelland (1990) and Williams et al (1990) adapted the polymerase chain reaction technique to produce anonymous genetic markers without the requirement for prior sequence information. Williams et al. (1990) called their markers RAPDs for Random Amplified Polymorphic DNA, and Welsh and McClelland (1990) called their system AP-PCR for arbitrarily primed PCR. In both systems a short oligonucleotide primer is used to initiate synthesis at many sites in the genome. If two sites are adjacent and in opposite orientation, amplification of a band will occur, and the length of the band will be defined by the distance between the sites. Products of the amplification are readily detected by gel electrophoresis. Many bands show genetic polymorphism and therefore can be used as genetic markers. Markers can be highly heritable and reliable under well controlled conditions (Tingey et al 1992; Thormann and Osborn 1992; Weeden et al 1992; Caetano-Anollés et al. 1992; Skrotch et al. 1992; Kesseli et al. 1992; Grattapaglia et al. 1992; Gepts et al 1992; Smith and Chin 1992). Careful control of reaction parameters is essential because amplification reactions are extremely sensitive to initial conditions.

The major advantage of PCR based markers was that they did not depend on prior knowledge of DNA sequence and they required only nanogram amounts of DNA. The amount of DNA required for PCR based markers is about 1000 fold less than that required for RFLP analysis (10 micrograms per restriction digest). The low requirement for DNA makes possible analysis of the

haploid tissue in the megagametophyte. In concept, PCR based markers have many of the advantages of isozymes, are more technologically accessible than RFLPs because they do not require cloning or molecular hybridization, and they are potentially automatable. Although the methods are very similar, RAPD markers were used more widely than AP-PCR. Both types of markers were dominant, and differed from RFLP and isozyme markers that were typically codominant. The theory and methodology of RAPD markers has been extensively described, applied and reviewed (Williams et al. 1992, Tingey et al 1992; Thormann and Osborn 1992; Weeden et al 1992; Caetano-Anollés et al. 1992; Skrotch et al. 1992; Kesseli et al. 1992; Grattapaglia et al. 1992; Gepts et al 1992; Smith and Chin 1992).

The question of dominance: Most genetic analysis in plants and animals has been carried out with codominant markers. These types of markers are common in isozymes, RFLPs and many PCR based markers. RAPD markers are dominant and therefore are usually not informative when it is necessary to distinguish heterozygotes from homozygotes. This property has caused many geneticists to avoid RAPD markers and some consider dominance to be a "substantial practical impediment" (Strauss et al 1992). Our approach has been different. We have chosen to prescreen RAPD markers for those that are informative, as a result greatly increasing the genetic resolving power of the method. It is possible to convert certain RAPD markers to codominant markers either by restriction digestion after amplification or by the extension of the RAPD primers after sequencing the ends (Kesseli et al. 1992). So far we find it easier to screen large numbers of potential primers for informative dominant markers and to ignore the other markers than to convert RAPDs to codominant markers.

The PCR-based technology led to RAPD genomic maps in many conifers and hardwood forest trees. The advantages of RAPD markers made possible the construction of genomic maps from individual trees in a relatively short period of time (Tulsieram et al. 1992; Grattapaglia et al. 1991, 1992; Neale and Sederoff 1992; Hoey et al. 1992; Jermstad et al 1992; Song and Cullis 1992; Teasdale et al personal communication; Nance et al 1992a, van Buijtenen 1992; Kubisiak et al 1992; Nelson et al 1992b). The species included loblolly pine, slash pine, long leaf pine, Monterey pine, slash pine/caribaea hybrid, yellow poplar and several species of Eucalyptus. The methods allowed the integration of the concepts of isozyme analysis with the power of DNA technology, particularly in conifers where genetic analysis could be done on the haploid megagametophyte. Maps of moderate marker density (less than 10 cM average density) could be constructed with approximately 100 primers and 10,000 PCR reactions. Such linkage maps described the maternal genome only, but were relatively detailed, and contained inferred linkage phase. Perhaps the most important result was that maps could readily be made for individual trees directly from a small number of open pollinated seeds. The open pollinated mapping strategy is the same one used to order isozyme loci on the early conifer linkage maps (Conkle 1981).

The construction of linkage maps comes from the 1:1 segregation of heterozygous markers in a tree used as the maternal parent. Analysis of the genotype is made on DNA obtained from the haploid megagametophyte that is expected to segregate either the dominant allele (+) or the null allele (-). Cosegregation analysis of a large number of heterozygous loci provides assignment of loci to linkage groups either by a Chi square test or using

MAPMAKER (Lander et al 1987). MAPMAKER also provides the order of the loci within a linkage group. .

Mapping in diploid full sib crosses. The simplicity of the haploid mapping system in the conifer megagametophyte can be approached for mapping with RAPD markers in diploids. The strategy involves the selection of a subset of informative markers from a larger number of markers. In a cross between two highly heterozygous and genetically distant individuals, many dominant markers will be heterozygous in one parent and null in the other. In such a case, segregation will be 1:1 just as in mapping with the haploid megagametophyte. The mapping strategy now referred to as a double pseudotestcross and has been applied to different types of markers in several species (e.g. Carlson et al. 1991; Ritter et al. 1990, and Weeden et al. 1992). Screening for such RAPD markers is relatively easy using both parents and a small set of six to eight progeny. The frequency of such informative markers can be high.

In a cross between *Eucalyptus grandis* x *E. urophylla*, 305 primers were screened. Of these, 151 were used to amplify 571 polymorphic markers distributed in two maps. The major advantage of RAPD markers resides in the ability to screen a very large number of potential markers easily, and to select a subset that are informative. A double pseudotestcross strategy can potentially be applied to diploid conifers as well as hardwoods. Only one generation of parents and progeny are needed. The strategy makes detailed mapping possible for a large number of trees currently being studied in breeding programs.

Mapping in open pollinated diploids: A theoretical approach has been proposed for mapping in open pollinated diploids. If rare dominant alleles are present in a heterozygous state in a maternal parent, segregation in diploid progeny will be 1:1. RAPD marker screening makes it possible to find large numbers of rare alleles if the levels of heterozygosity are high. Simulations show that rare dominant alleles that have frequencies at least as high as 0.2 in the general pollen pool can still be useful as markers for mapping and for QTL analysis.

Dominant markers for targeted mapping (bulking). An important variation in mapping that involves pooled samples is particularly useful with dominant markers. This procedure called "bulked segregant analysis" (Arnheim et al 1985; Michelmore et al. 1991) allows the identification of markers flanking a defined locus without mapping of the entire genome. For example, an isozyme locus could be mapped by pooling 10 samples for each allele in a segregating population and preparing two DNA samples. The two samples are screened for primers that are present in one pool and absent in the other. In this way markers are detected that are linked to specific alleles. In a situation where a map already exists, bulking can quickly place new genes on a map or alternatively can be used to saturate a specific region for fine structure mapping. In a targeted mapping study in peach (*Prunus persica* L. Batsch) 15 markers were found linked to either the Gr (red leaf) locus or the Mdh1 locus on linkage group 5 (Chaparro et al. 1993). Bulking is also applicable to saturation mapping for a specific chromosomal interval (Giovannoni et al 1991).

In theory, bulking can be used in systematics to find dominant markers that will serve to discriminate between species. O'Malley et al (1992) screened 200 primers and found 84 primers and 129 RAPD fragments that provided informative polymorphisms in a study of the relationships of Central American pines.

The main impediments to use of DNA markers for forest genetics that were perceived in 1991 have been lessened to a great extent. PCR based markers reduce direct costs, do not require clone banks, and eliminate the cumbersome technology of molecular hybridization. Theory and technology are now available to utilize the high levels of diversity and heterozygosity in forest trees for genomic mapping. Much of the genetic material in forest tree breeding programs is now accessible to genome mapping. Three kinds of mapping strategies are available, using haploid megagametophytes of open pollinated conifers, the pseudotestcross strategy for full sib crosses between diploids, and the "rare allele" strategy for mapping in an open pollinated diploid.

Mapping in any individual tree can be a powerful tool for quantitative trait dissection. If we now assume that it is relatively easy to map, how can mapping be applied to practical problems of tree breeding? The first level of application is the dissection of quantitative traits. For most traits of commercial importance in forest trees, the genetic structure underlying the phenotype is not understood. For decades, it has been assumed that most traits were controlled by many genes, being polygenic rather than oligogenic. Breeding strategies based on quantitative genetic theory of polygenic inheritance were highly successful in a wide variety of plants and animals. Genetic mapping now makes possible experimental testing of oligogenic versus polygenic inheritance in forest trees through genetic dissection of quantitative traits.

Bush and Smouse (1992) used allozyme polymorphisms to relate yield and fitness components in forest trees with a strategy similar to that used with DNA markers for QTL analysis, but did not find strong correlations. As Strauss et al (1992) argued, with increased genetic resolution, the architecture of quantitative traits affecting yield and fitness can be approached with unprecedented precision. However, the absence of adequate methods and theory led Strauss et al. (1992) to a negative view of the potential for quantitative trait dissection. Strauss et al. 1992 argued that quantitative trait dissection in agronomic crops is of little relevance to forest tree populations, and that the near term usefulness of QTLs to tree breeding will be limited. Most studies of agronomic crops are based on inter or intra specific hybrids, and many of the species studied have been under domestication for hundreds or thousands of years.

It may be argued that domestication and inbreeding would make QTLs hard to find in agronomic crops. Nevertheless, quantitative trait loci abound in agronomic crops (Paterson et al 1990; 1991; Stuber et al 1987; Stuber 1992; Tanksley et al 1989; Kiem et al 1990). It can easily be argued that forest trees, as essentially undomesticated plants, would be rich in quantitative trait loci, particularly related to traits associated with growth and environmental adaptation. The immediate problem therefore, is the design of adequate tests to define QTLs in forest trees.

The design of adequate tests for QTLs should be different for forest trees than for crop species because of the different mapping strategies. QTLs are defined by statistically significant associations or correlations of specific markers (regions of linkage maps) with major components of a quantitative phenotype. A specific strategy, within family open pollinated analysis, can be used for QTL analysis in conifers using the haploid megagametophyte. For QTL analysis in full sib diploid crosses, traditional methods of maximum likelihood have been applied (Lander and Botstein 1989).

Quantitative trait dissection using within family analysis of open pollinated conifers.

The special genetic features of the haploid conifer megagametophyte makes possible tests for QTLs in open pollinated progeny from single trees. A single haploid megaspore gives rise to the maternal contribution to the embryo and to the megagametophyte. The genotype of the maternal nucleus before fertilization is identical to the megagametophyte. Therefore, megagametophytes rescued from germinating seeds can be used for genetic mapping, and the germinants can be scored for a quantitative phenotype. Correlation of maternal markers with the phenotype is equivalent to other kinds of QTL analysis, except only correlations of the maternal genotype with the trait is tested. Several such experiments are in progress.

An unusual feature of QTLs identified by within family analysis of open pollinated maternal trees, is that they would be detected in a mixed genetic background contributed by many different pollen parents, rather than a single parent as in a full sib cross. Such QTLs would not be cross specific, but would have general combining ability. QTL analysis in full sib crosses in diploids using the pseudotestcross is reduced to that of a standard backcross. Two sets of analysis are done, one for each parent, as it is a double pseudotestcross.

Adequate tests for QTLs, with open pollinated progeny may require large sample sizes and genotyping of many markers. Experiments in progress use sample sizes of approximately 1000, with the expectation that about 100 markers will be screened. Although sampling from the tails of the distribution would reduce the number of reactions required, it is necessary, at least in these early stages to expect to be able to process 100,000 reactions in a series of experiments. To do this it is necessary to do PCR reactions at a scale of one to two thousand per day. We have approached this problem by using a microtiter plate format (96 wells), multiple thermocyclers, a robotic pipetting station, and video still imaging for recording gel data, and data analysis, using MAPMAKER (Lander et al 1987), and GMEDEL (Liu and Knapp 1992a) on Macintosh computers and a Sparc II workstation. QTL analysis is done with MAPMAKER/QTL (Lincoln and Lander 1989) and QTLSTAT (Liu and Knapp 1992b). Similar automation of RAPD reactions has been set up by Nelson et al (1992).

Identification of QTLs provides predictive hypotheses to test in breeding. QTLs are statistically defined and have the potential to be artifacts. What is needed to verify the biological reality of a QTL?. The detection of a QTL provides a predictive hypothesis to test in independent breeding experiments. Markers associated with QTLs in specific crosses or in open pollinated progeny should predict phenotype or performance. Identification of QTLs is only the first step in QTL analysis. Predictive tests will be the second stage of QTL experiments with forest trees. The best test of the putative QTLs will be in

actual breeding experiments, many of which are currently available for analysis. Verification will come from repetition, examination of related families, and retrospective analysis as well as prospective analysis.

What is really needed to use markers in breeding? The major limitation to tree breeding is the time required for one cycle of breeding and selection. The most valuable contribution that markers could make to breeding would be to save time by shortening the time before selection or to reduce the number of breeding cycles. This can be done in two ways, either by the early identification of superior progeny or by the identification of parents that will yield superior progeny. In contrast to annual crops where phenotypic selection can be carried out in weeks or months, woody plants may not show a mature phenotype for many years.

Identification of QTLs: Evidence is accumulating in several laboratories to identify QTLs for important traits in forest trees. Neale and coworkers have identified loci influencing wood specific gravity in loblolly pine (this conference) with a view toward marker aided selection (Williams and Neale 1992). Bradshaw and coworkers using hybrid poplar have identified loci for phenology and growth characteristics (Bradshaw, personal communication). In our group, QTLs for volume growth at rotation age have been identified in an open pollinated family of *Eucalyptus grandis*. In a full sib pseudotestcross of *E. grandis* and *E. urophylla*, QTLs for micropropagation ability (fresh weight of shoots in culture) have been identified. Even though QTL analysis is at an early stage, it would appear that association of markers with traits of significant biological effect or of commercial value can be readily detected.

Additional QTL experiments in progress: In loblolly pine, markers are being used to dissect the oligogenic nature of fusiform rust disease in progeny from a series of crosses challenged with single aeciospore lines of fusiform rust. The genetic structure of resistance to rust is of interest as a "threshold" trait and because it provides a test case for marker aided selection. As argued by Lande and Thompson (1990), Strauss et al., (1992), marker aided selection would be most efficient for a trait with relatively low between-family heritability. A general framework for the inheritance of resistance to rust in southern pines has been described (Kinloch and Walkinshaw (1991) and an approach to genetic analysis using advanced pedigrees for rust resistance has been presented (Nance et al. 1992b). Nance et al (1992) have also described strategies for use of marker aided selection for rust resistance in advanced pedigrees.

A series of experiments are in progress to analyze quantitative components of early height growth in loblolly pine using the within "half sib" approach. Cyclic shoot growth will be measured in open pollinated seedlings and correlated with maternal markers. QTL analysis will be carried out to identify regions of the genome that explain significant components of the early cyclic growth trait. Shoot elongation during first year is strongly correlated with field performance at 8 years (Bridgwater et al 1985; Williams 1987; Bridgwater 1990). The trait has high heritability and a strong juvenile mature correlation (Li et al 1991). A similar series of studies have been started to correlate variation in rooting ability within families with molecular markers by a similar analysis. Analysis of progeny from open pollinated trees is particularly appropriate and useful for coniferous forest trees. Half sib approaches to quantitative trait dissection using molecular markers have been

proposed for animal genetics (Haley 1991) where progeny genotypes are used to deduce the contribution of the common male parent.

The relationship of markers and breeding proceeds through three steps:

1. Construction of individual maps for important trees in a breeding program. Breeding applications will require many specific maps. The objectives for many breeding programs would be the construction of individual tree specific maps that had reasonably complete coverage at the 10 to 20 cM level. A single high density map for the species would be required to provide anchor points for the single tree, cross specific maps. Anchor points are essential to determine the relationship of QTLs localized in different trees.

2. Individual tree maps would be used in progeny tests to locate quantitative (QTLs) and qualitative genes of interest. Lethal or semilethal loci could be located and identified in specific trees. This information would provide the data base for the future generations of breeding and selection for each specific breeding population.

3. Use of specific markers in association with specific traits in genome map assisted plant breeding (GMAPB) (Liu and Hayes 1992). GMAPB would employ a new generation of computer software designed to use markers for marker aided selection, and in the design of breeding strategy. Selection of parents based on genomic mapping information would be particularly valuable for optimizing gain in selection experiments. Choosing parents with different QTLs contributing to the same phenotype would provide greater opportunity for increased gain. Breeding of trees with mapped lethal factors could lead to the establishment of inbred lines, and allow the development of new breeding strategies for forest trees.

Other aspects of genomic mapping: In addition to applications to breeding, genomic mapping in trees now has reached a level of technology so that it is possible to consider the targeting of important genes for map based cloning even for conifers. Fine structure maps based on RAPD markers are a first step to fine structure physical maps around important genes. It is not too early to begin such experiments. We are not at a stage where the identification of a specific gene in a well mapped interval is feasible, but this component of map based cloning should become an intense area of research, even in forest trees.

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CHALLENGES FOR INSECT PEST MANAGEMENT IN FOREST TREE SEED ORCHARDS

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Abstract.--There are large applied forest tree improvement programs for Douglas-fir in the Pacific Northwest and loblolly pine in the South. There are also similar programs for tree species of lesser commercial importance. Intensively managed seed orchards, which produce tons of genetically improved seed used to reforest millions of acres of commercial forest land, are key elements of these programs. Insect pests are a very serious problem in seed orchards. In the 1970's and 1980's researchers developed insect control methods that were quickly implemented by pest management specialists and orchard managers. As a result, seed yields exceed expectations. The 1990's bring new challenges for seed orchard pest management. New challenges include (1) insuring that insecticides are available for use in seed orchards, (2) reducing insecticide loads in orchards, (3) improving efficiency of control tactics, (4) addressing changing orchard management goals, (5) considering insect pests in orchard site selection, (6) gaining a better understanding of interactions among arthropod species in the seed orchard canopy, and (7) developing noninsecticidal tactics and strategies for controlling insects. Ways of addressing these challenges are discussed.

Keywords: Coneworms, seed bugs, insecticides, cone and seed insects.

INTRODUCTION

Seed orchards are an important part of the applied tree improvement programs in North America (Zobel and Talbert 1984) and are key elements for the success of these programs. While less than 20,000 acres of orchards exists in North America, these areas represent a major forestry investment. Individual orchards are small, ranging in size from 5 to 500 acres. Management is for the single purpose of supplying the tons of genetically improved seeds needed to grow seedlings for the reforestation of millions of acres of commercial forest lands. An array of insect pests threatens seed crops (Ebel et al. 1976, Hedlin et al. 1980, Turgeon and deGroot 1992). Each tree species has its own unique complex of cone and seed insect pests.

As first-generation seed orchards came into production during the 1970's the demands for genetically improved seed were high. Once the impact of cone and seed insect pests was clearly recognized, entomologists developed insect control methods and orchard managers quickly put them into practice (DeBarr 1990). These methods were highly effective and yields exceeded expectations of tree improvement specialists and geneticists.

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Wakeley (1954) noted that in harvests from natural stands of southern pines, "each species averages about 1 lb. per bushel of cones in good years, about 0.5 lb. per bushel in years of moderate crops and 0.2 lb. per bushel or less in very poor crop years". Twenty years later, a loblolly pine seed orchard in the North Carolina State University-Industry Cooperative yielded 2.36 lb. per bushel (Anon. 1985). The report stated that "effective orchard management practices have allowed cooperative members to reach production efficiencies once thought impossible".

The theme of our 1993 Southern Forest Tree Improvement Conference (SFTIC) is "Forest Genetics in a Changing World". The 1990's bring a new series of challenges for seed orchard pest management. I discuss some of these challenges and ways to meet them in this paper.

INSURE INSECTICIDE AVAILABILITY FOR SEED ORCHARDS

Insecticides are the most widely used method for controlling cone and seed insects in forest tree seed orchards. They will continue to be important for seed orchard pest management, as long as they remain available to us. Insecticides most effective for cone and seed insect controls have long residual or systemic activity. They are readily available, easy to use, cost-effective and provide broad-spectrum control of many different cone and seed insect pests. Both managers and the general public are also aware of their potential disadvantages. To ensure that insecticides are available for our use, tree improvement specialists and forest entomologists must work together to keep our current registrations and register any new insecticides that are potentially useful for controlling orchard pests.

Retain Current Registrations

Few insecticides are registered for use in southern pine seed orchards (Table 1). Most of the registrations are at least 10 years old (van Buijtenen 1981). The Environmental Protection Agency (EPA) granted the last Federal registration in 1987 and canceled the use of Furadan® in seed orchards in October 1992. Other registrations are being reviewed by the EPA and some of these insecticides may not be re-registered by the chemical companies. Recently, the SFTIC organized a subcommittee called the Seed Orchard Pest Management Committee (SOPMC) to address this problem. Working together, this group of tree improvement specialists and forest entomologists made several important accomplishments. One is the reclassification by EPA of seed orchards from forestry sites to non-food crop, terrestrial sites. This action should make it somewhat easier to keep insecticides available for use in seed orchards.

Table 1. Federal registrations of insecticides for cone and seed insect control in seed orchards.

Trade name	Common name	Class*	Year registered
Cygon	dimethoate	OP	1962
Guthion	azinphosmethyl	OP	1974
Furadan**	carbofuran	CB	1976
Ambush	permethrin	PY	1980
Pounce	permethrin	PY	1980
Pydrin	fenvalerate	PY	1980
ASANA***	esfenvalerate	PY	1987
Foray	Bt	MC	1991
Capture	bifenthrin	PY	199?

*OP=organophosphate, CB=carbamate, PY=pyrethroid and MC=microbial insecticide.

Canceled by EPA Oct., 1990. *Isomer of Pydrin.

Register New Insecticides

Only a few new insecticides have become available in recent years and chemical companies no longer eagerly pursue registrations for forestry uses. These markets are small and there is increasing public concern with the use of insecticides on forest lands. Efficacy data for early registrations of insecticides for cone and seed insect control in seed orchards were based upon field tests using individual trees. However, the current method of choice for applying insecticides in seed orchards is with aircraft. Region-wide efficacy tests of aerial applications of new insecticides are costly and difficult to carry out. Recently, the SOPMC committee conducted a Southwide efficacy test of the pyrethroid insecticide, Capture. Some of the problems were the uncertainty as to which formulation the manufacturer wanted to test and register for seed orchard use, a year delay because of an Experimental Use Permit and limited replication due to the small number of orchards suitable for the test. In addition, all the time and resources for planning and conducting the test were contributed by members of the SOPMC and the participating orchard managers. Finally, standardized procedures had to be developed and orchard personnel from each site had to be trained to ensure consistency in the applications. Because of the efforts by the SOPMC, it is now permissible to use Capture in seed orchards, under 24-C registrations, in most of the southern states (Lowe et al. 1993). If registered by the EPA, Capture will be the first new Federal registration of an insecticide for seed orchard use in almost a decade.

MINIMIZE INSECTICIDE LOADS IN SEED ORCHARDS

Continuing to reduce the amount of insecticide applied in seed orchards will help to keep insecticides available for our use. Additional benefits include lower costs, increased

safety, reduced environmental risks and delayed development of pest resistance. Two ways to reduce insecticide loads in seed orchards are to use less per application and make fewer applications.

Reduce Insecticide Rates per Acre

Using less insecticide per acre diminishes both costs and potential environmental problems. The rates we use today, are much lower than those once applied for cone and seed insect control in seed orchards (Table 2). This is the result of two important changes in pest management that occurred in the early 1980's.

Table 2. Insecticide amounts per acre for single applications with several control methods.

Insecticide	Control method	lb.active ingredient/acre*
Furadan	Soil systemic	12**
Guthion	Hydraulic sprayer	8
Guthion	Mistblower	5
Guthion	Aircraft	3
Ambush/Pounce	Aircraft	0.75
ASANA	Aircraft	0.19
Capture	Aircraft	0.1

*assuming the maximum registered rate. **assuming 48 trees 10" dbh/acre.

First, the pyrethroid insecticides, Ambush® and Pounce®, were registered for seed orchards. These pyrethroid insecticides are more effective, on an active ingredient per acre basis, than is the older organophosphate insecticide, Guthion®, or the carbamate insecticide, Furadan®. ASANA® is a refined isomer of Pydrin®. Capture® is a second-generation pyrethroid, which is effective at even lower rates than Ambush®, Pounce® and ASANA®. We must continue to look for new insecticides that work at even lower rates.

Second, the use of aircraft has made it possible to get the insecticide to cones in the tops of the trees more efficiently than with ground sprayers (Barry et al.1984). Using aircraft often attracts more public attention, but it is a much better choice than ground applications. Besides lower rates per acre, other advantages include better spray coverage, reduced worker exposure, better timed applications and lower costs because of improved efficacy. It is particularly important that we retain the option of using aircraft to apply chemical and microbial insecticides in seed orchards. In the future, aircraft may also be needed to apply chemicals that modify insect behavior.

Reduce Application Frequency

Fewer applications per year mean less pesticide load in seed orchards. It was not uncommon to spray orchards as often as 6 times each year, when genetically improved seed was scarce. Since the importance of each pest often varies with the orchard site, orchard managers have learned how frequently they must spray to protect seed crops in their particular orchard. With cone crop monitoring, surveys using pheromone traps and their own individual experience, the number of applications per year is more likely to be 2 to 4.

DEVELOP STRATEGIES FOR CHANGING ORCHARD MANAGEMENT GOALS

Low Intensive Management in Older Orchards

As new generation orchards become productive, pest management activities are often stopped in older orchards. However, some managers continue to harvest seed from the best clones in their old orchards. Without insect control yields will be poor. Spraying the entire orchard is not a cost-effective alternative. In this situation, individual tree protection appears to offer some advantages. The idea of controlling insects on individual trees in seed orchards is an old one (DeBarr 1971), but it never gained acceptance because the demands for seed were high. Any of the insecticides currently registered for use in mistblowers or hydraulic sprayers will protect individual trees. Systemic insecticides implanted into the trunks of pines is an effective way to control cone and seed insects (Merkel and DeBarr 1971). However, there are no systemic insecticides registered for use as implants in southern pine seed orchard trees.

High Intensity Management in Young Advanced Generation Orchards

Seed from advanced generation orchards are always scarce. Insect control in these orchards is essential and managers are unwilling to tolerate losses. Since yields from first generation orchards exceeded expectations, geneticists are establishing smaller orchards for advanced generations. Continued high yields, will depend upon pest management that is as good as or better than that for first-generation orchards.

ORCHARD SITE AND INSECT PEST MANAGEMENT

Geneticists consider many factors in selecting a site for a new seed orchard. Usually, not much thought is given to the effect of the location on future insect pest management. Selecting the wrong site can result in constant problems with insect pests, as well as limit the options for pest management. Guidelines for evaluating an orchard site for pest management problems would be a valuable aid.

Minimize potential for orchard infestation and reinfestation

Advanced generation orchards established next to older orchards, with large cone-bearing trees, will quickly become infested by cone and seed insects. They are also highly susceptible to reinfestation. Once harvesting has stopped in older orchards there is seldom any insect control. Abundant cones in these sanctuaries allow insect numbers to increase. Managers should destroy these orchards, if they cannot afford to control the pests. Invasion of a new orchard can also occur when cone-bearing trees are present in adjacent natural stands, plantations, abandoned fields, fence rows or parks and residential areas. Locations such as these should be avoided. If more than one tree species is planted at the same orchard site, problems are created if they share the same insect pests. For example, when a loblolly pine orchard is next to a slash pine orchard, seed bugs may concentrate on the loblolly pines, after the slash pine cones are harvested.

Avoid Environmentally Sensitive Sites

Problems associated with pest management practices, especially the use of insecticides, are often not considered when choosing an orchard site. Health, safety and environmental problems due to insecticide drift or runoff, whether real or perceived, can rule out the use of insecticides. There have been many cases where insecticides could not be used or had to be used with extreme caution because orchards were located too close to springs, wells, streams, rivers, lakes, homes, farms, or urban areas.

IMPROVE PEST MANAGEMENT TACTICS

With few exceptions, orchard managers apply insecticides on a preventive basis to control cone and seed insect pests. Two ways to make insect pest management more efficient are to develop methods for predicting the need for control and to time controls.

Develop Techniques to Predict the Need for Control

It is very difficult to predict losses caused by insects. Few successful examples exist for agriculture, fewer still for forestry, and practically none for seed orchards. One exception is the egg sampling technique for the Douglas fir cone midge (Miller 1986). Insect populations are affected by many biological and environmental factors and their interactions. Practical techniques for predicting losses must be reliable, inexpensive and easy to use. Cone and seed insects are particularly difficult to sample because low numbers cause substantial damage and they spend long periods of the time in life stages that are small and well hidden. These low numbers have a highly variable distribution within the orchard and the large spatial area of the tree crowns. To be most useful, prediction methods should be available for all the key pests for each host species. Otherwise, orchard managers will opt for using preventive sprays.

Develop Techniques for Timing Controls

There are a number of ways to time controls to coincide with periods of maximum vulnerability in the life cycle of an insect pest. A readily identifiable event in the phenological development of the host can be used. Degree-day models are based on the fact that insect growth is largely controlled by temperature. Temperature development relationships have been determined for the southern coneworm (Hanula et al. 1987). Studies of the temperate development relationships for the leaffooted pine seed bug and the shieldbacked pine seed bug are currently underway in our laboratory. The challenge is to demonstrate the practical value of degree-day models for pest management.

INCREASE KNOWLEDGE OF SEED ORCHARD ECOLOGY

Research on cone and seed insects has focused on the major cone and seed insect pests. However, we know relatively little about interactions among insect and other arthropod species found in seed orchards, the role of natural enemies in regulating pests, or potential for problems with secondary-insect pests. Understanding these biological details is essential to the development of new control strategies. Such knowledge can be the key to successful insect control and can prevent unforeseen problems. Two examples illustrate these points.

Observations of the webbing coneworm led to the discovery that young larvae feed in the catkins of loblolly pines before attacking second-year cones. We discovered that they were highly vulnerable to sprays applied within 7 days after peak pollen flight, just before they attack cones. Webbing coneworm control using this "7-day window of opportunity" is very reliable.

Outbreaks of secondary insect pests occurred when pyrethroid insecticides were first introduced for cone and seed insect control in southern pine seed orchards in the early 1980's. Pydrin caused the most severe problems. These outbreaks occur because Pydrin residues stay on pine foliage longer than for other insecticides (Nord and DeBarr 1992). This residual activity provides excellent control of cone and seed insects. It also kills the natural enemies of scale insects (Clarke et al. 1988), but not the scale insects. In contrast, Guthion was almost as toxic to the scale insects, as Guthion (Clarke et al. 1992).

DEVELOP NEW CONTROL STRATEGIES AND TACTICS

Seed orchards offer one of the most ideal situations in forestry to implement new approaches to insect pest management. Crop values are high and orchard sizes provide clearly defined areas for treatment. Skilled managers and rapid communication through the tree improvement cooperatives and the SFTIC, make technology transfer easy. Some new approaches that are potentially useful for cone and seed insect control include cultural control, pathogenic microbials, behavioral chemicals and biocontrol. As the following examples show, each approach has its strong and weak points.

Cold water sprayed on Douglas fir orchards prevents gall midge attacks by delaying female strobili development (Miller 1983). Limitations include the high costs for irrigation equipment and lack of control during years with cool temperatures. However, if dimethoate, the insecticide commonly used to control the midge is unavailable, this tactic might be more acceptable. Prescribed fire kills overwintering cone beetles in eastern white pine seed orchards. Since EPA rescinded the registration of Furadan in 1990, fire is the only alternative available for cone beetle control and it has been used in several seed orchards. Major limitations are adequate fuel, relatively few days with optimum condition for burning and concern over the effects of repeated fires on tree health..

The microbial insecticide, *Bacillus thuringiensis* (Bt) will control some of the coneworm species, however, Bt only affects certain insect groups and it will not kill seed bugs. There are many species of parasites and predators of cone and seed insect pests (Yates 1989), but we know little about their contribution to control in seed orchards. It seems likely that natural enemy populations are severely affected by the routine use of insecticides in seed orchards. Augmentation of natural enemies through rearing and release seems impractical, but less frequent use of more selective insecticides will conserve these potentially useful insects in seed orchards. The use of synthetic pheromones, attractants and inhibitors to modify insect behavior through such techniques as trap-out or male confusion offers promise, but much additional basic and applied research will be necessary to develop techniques that provide reliable cone and seed insect control. These chemicals are also subject to the same complexities of registration, as are traditional chemical insecticides.

Methods such as these, are likely to be less reliable and more expensive than chemical insecticides. Therefore, even if these approaches prove useful, they may not be widely used, as long as chemical insecticides are available. To compete with insecticides, new tactics and strategies must be cheaper, more effective, offer environmental advantages or be easier or safer to use than the currently registered insecticides.

CONCLUSIONS

There have been many challenges for insect pest management in forest tree seed orchards during the past 25 years. The formidable challenges we face today are even more complex than those that confronted us before. Research will lead to the discovery of new and better pest ways for dealing with cone and seed insect pests. However, there is a wide gap between the promise of research and practical pest management techniques. Continued cooperation by tree improvement specialists and forest entomologists is necessary to bridge this gap and ensure that orchard managers have the tools they need to produce the large quantities of seed for forestry in the South.

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POSTER SESSION

EFFECT OF COLD TREATMENT ON CONVERSION OF BLACK LOCUST SOMATIC EMBRYOS

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The objective of this study was to determine the effect of cold treatment on the conversion of naked or encapsulated black locust (*Robinia pseudoacacia* L.) somatic embryos. The experiment was carried out using one embryogenic line established from an immature seed (Merkle and Wiecko 1989, Merkle 1992) and maintained in F medium (10A40N; Finer and Nagasawa 1988) supplemented with 3 mg/l 2,4-D. Globular stage somatic embryos were obtained by transferring approximately 0.5 g of proembryogenic masses (PEMs) to hormone-free liquid F medium. Cotyledonary stage embryos were obtained when the globular stage embryos were plated on the same medium solidified with agar. For encapsulation, cotyledonary embryos were placed in a beaker of 2% sodium alginate solution, either dissolved in F medium or distilled water. Then, drops of alginate solution containing individual embryos were pipetted into a beaker of 50 mM calcium chloride solution forming a calcium alginate bead around each embryo. Naked and encapsulated embryos were stored on moist filter paper at 4°C for 0, 15, 45 or 100 days, after which they were tested for germination on half-strength MS medium (Murashige and Skoog 1962). With no cold treatment, 71% of the naked embryos versus 41% of the encapsulated (either in water or F medium) developed into plants. Fifteen days of cold treatment increased germination rates up to 95% for naked embryos, 80% for embryos encapsulated with 2% sodium alginate in water and 75% for embryos encapsulated with 2% sodium alginate in F medium, respectively. Cold treatments longer than 15 days resulted in low germination percentages. Recovered plants were acclimatized and grown in the greenhouse.

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THE RAIN (REMOTE AUTOMATED INTELLIGENCE NETWORK) COMPUTER SYSTEM FOR SEED ORCHARDS

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Abstract.--RAIN is a computer network that monitors environmental variables and provides an electronic message system among users. Stations in the network use portable computers equipped with sensors that record local variables including temperature, leaf wetness, rainfall, wind speed and wind direction. These stations are highly reliable and very inexpensive. The network's central computer hub provides users with data bases, models and site-specific pest control recommendations. RAIN stations are in operation in more than twenty seed orchard sites throughout the eastern United States. The system is a useful tool for research and management. RAIN automatically advises managers when to spray insecticides to control the Nantucket pine tip moth (*Rhyacionia frustrana* Comstock). The recommendations are site-specific electronic messages based upon local temperature data collected by RAIN and on historical averages. Similar degree-day models are being developed to aid in the control of several cone and seed insects. Procedures have also been developed to use RAIN to collect pheromone trapping data from the Southwide coneworm survey.

Keywords: Temperature, rainfall, wind, Insecticides, Nantucket pine tip moth, coneworms, seed bugs, degree-day models.

RAIN is a network of on-site personal computers connected to a central hub consisting of two mini-computers located in the Entomology Department at the University of Georgia, Athens, GA. (Pickering et al. 1990). It monitors environmental variables and provides an electronic message system among users in both research and management. RAIN stations are installed at intensively managed agricultural and forestry sites throughout the southeast (Fig. 1). These stations link seed orchard managers with scientists, Forest Pest Management Specialists and Cooperative Extension personnel.

Each station in the network is an inexpensive portable computer, which is equipped with sensors that record local variables including temperature, leaf wetness, rainfall, windspeed and wind direction. Other types of sensors can also be added. Depending upon the variables monitored, each station costs \$500 to \$1500. Non-dedicated telephone lines are used for communication. Messages and sensor data are exchanged during unattended nightly telephone calls to the hub that take 1-3 minutes. Messages can be addressed to specific individuals who can save, delete, reply or forward them.

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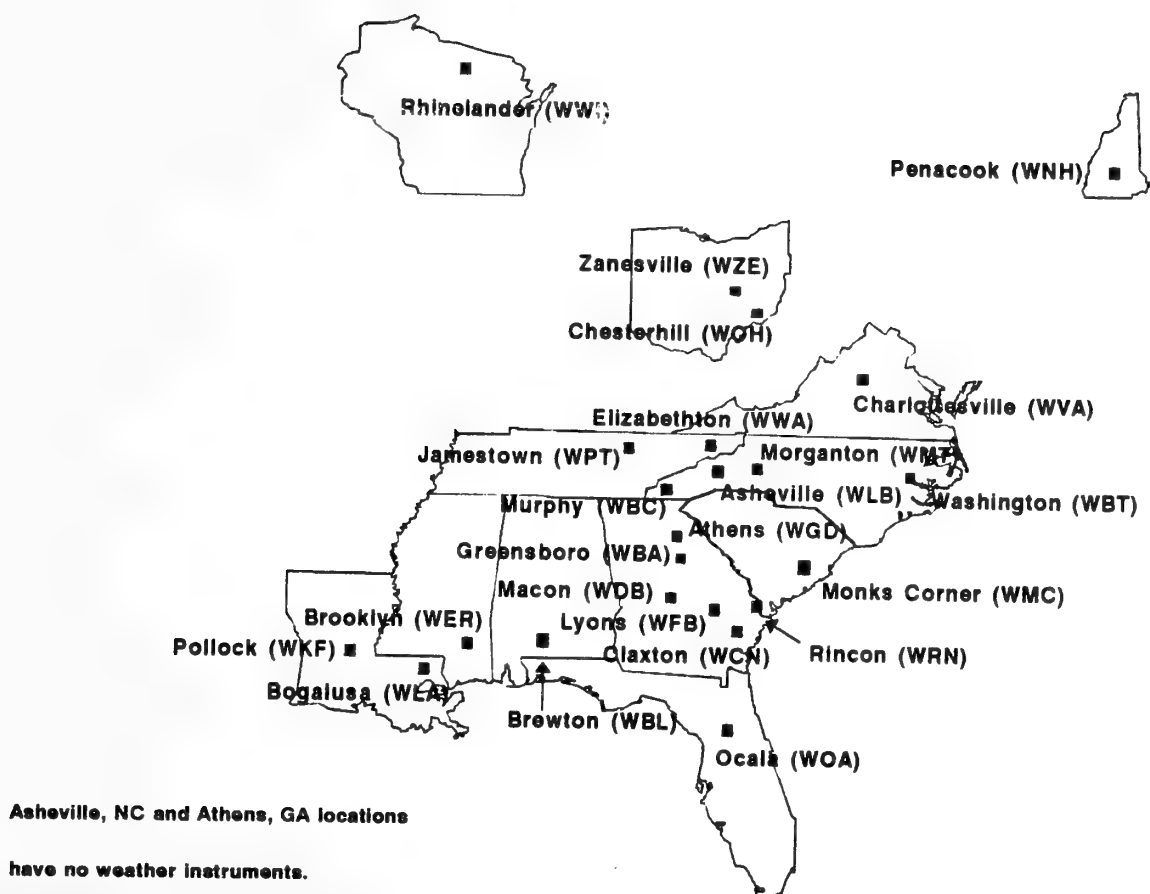


Figure 1. RAIN station locations at seed orchards in the eastern United States.

The network's central hub can provide users with databases, models and an expert system. The expert system sorts and forwards incoming information. Four types of information are collected from remote stations: (a) hourly sensor readings, (b) messages initiated by orchard managers (c) replies to electronic surveys sent from the hub, and (d) diagnostics. These data are accessible via microcomputers with modems and are used in both research and management.

We are developing RAIN for regional monitoring and management in forest tree seed orchards. Orchard management costs will be reduced by sharing data. Forest Pest Management specialists will be able to post electronic pest advisories based on historical data and up-to-date pest surveys. Procedures have been developed for incorporating the Southwide Coneworm Survey data into the Rain network (Mangini et al. 1993). This will eliminate the need for sending forms through the mail and automated database updates and summaries will be possible. Managers can forgo the use of pheromone traps until notified to start trapping for a specific coneworm species. They can be rapidly alerted to unusual pest outbreaks.

Currently, a temperature based phenology model for the Nantucket pine tip moth (*Rhyacionia frustrana* Comstock) is operational (Pickering et al. 1989). This model uses catches in pheromone traps, historic information on seasonal temperatures and temperature data updates collected by the network. It calculates optimal spray dates, thus reducing the number of chemical sprays required to protect progeny tests, newly established seed orchards or Christmas tree plantations. Short, site-specific messages advise managers when to apply insecticide to control this pest.

We plan to implement other pest models on RAIN. Threshold temperatures and degree-day estimates have been determined for the southern pine coneworm (*Dioryctria amatella* Hulst...) eggs (Hanula et al. 1984) and larvae (Hanula et al. 1987). Degree-day models based upon these data have been field tested and resulted in control with two applications that was as good as that obtained with four monthly applications (G. L. DeBarr and J. L. Hanula--unpubl. data). Threshold temperatures and degree-day estimates have also been determined for the shieldbacked pine seed bug (*Tetyra bipunctata* H. & S.) and the southern pine seed bug (*Leptoglossus corculus* Say) (J. C. Nord and G. L. DeBarr--unpubl. data).

RAIN a reliable, inexpensive computer network . It has many valuable functions and will serve as the keystone for future pest management systems in seed orchards.

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Mating Patterns and Pollen Dispersal in Knobcone Pine.

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Mating system and effective pollen dispersal in a natural stand of knobcone pine (Pinus attenuata Lemmon.) were studied using eleven isozyme loci. Analyses were performed by fitting neighborhood and mixed-mating models to multilocus genotypic arrays of offspring from four mother trees. Average individual tree outcrossing rates were estimated to be 0.984 and 0.964 for the respective models. The proportion of offspring sired by males outside the neighborhood of each mother tree (i.e., outside a radius of 15 m) was 0.55. Thus about 43% of the mating were due to outcrossing with nearby males (about 75 within each neighborhood). The neighborhood model makes it possible to evaluate the importance of direction and distance to female parents, as well as relative fecundity, on the mating success of paternal trees.

DETECTION OF A HEIGHT GROWTH LOCUS IN
AN F2 MAPPING POPULATION OF *Prunus persica*

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An F2 mapping population was generated by self-pollinating the *Prunus persica* clone 'Georgia Belle'. The F2 population generated was segregating for several morphological traits and the isozyme malate dehydrogenase (*Mdh1*). Linkage between the *Mdh1* locus and a locus controlling height growth was detected. Homozygous *Mdh1*-2 trees were significantly taller than *Mdh1*-1 homozygotes after one year of growth. Fine structure mapping around the *Mdh1* and height growth loci will be performed using the bulked segregant analysis technique and RAPD markers.

ISOLATION OF DNA FROM FOURTEEN HARDWOOD TREE SPECIES AND AMPLIFICATION USING RAPD TECHNOLOGY

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A CTAB extraction method was used to isolate DNA from leaf samples of fourteen mature and five juvenile hardwood tree species. Species studied included six oaks and two hickories, as well as tulip poplar, sugarberry, dogwood, persimmon, blackgum and swamp tupelo.

DNAs isolated from samples collected in spring and late summer from the same source were compared to determine if amplified products remained consistent over season and physiological state. Additional phenol:chloroform:isoamyl alcohol (PCIA) purification was usually necessary to treat the DNA before it could be amplified using random primers that detect polymorphic DNA (RAPD) markers. DNA samples, before and after PCIA purification, were amplified and compared to determine if purification affected the integrity of the DNA. Extraction procedures and amplified products of DNAs isolated from leaf samples of mature trees and juvenile seedlings of the same species were compared.

Further studies were conducted on the masting (acorn, nut, seed) phenomenon for several of the oak species using bulk segregant analysis methodologies.

AN UPDATE ON THE SOUTHWIDE CONEWORM SURVEY

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Abstract.--Since 1981, sticky traps baited with synthetic pheromones have been used in seed orchards across the South to monitor the activity of four species of coneworms: *Dioryctria amatella*, *D. clarioralis* (Walker), *D. disclusa* Heinrich and *D. merkei* Mutuura and Monroe. The Southwide Coneworm Survey provides site-specific and regional information on insect distribution, abundance and seasonal activity which can be used in making pest management decisions and planning research studies.

Keywords: Cone and seed insects, *Dioryctria*, insect abundance, insect distribution, insect sampling, IPM, pest management, pheromone traps.

INTRODUCTION

Since 1981, a survey has been conducted using sticky traps baited with synthetic pheromones to monitor populations of four species of coneworms in pine seed orchards throughout the South. The survey is a cooperative effort between the USDA Forest Service Southeastern Forest Experiment Station (SEFES); USDA Forest Service Forest Pest Management - Region 8 (FPM); and cooperating industry, state and federal orchards. The Southwide Coneworm Survey provides site-specific and regional information on the distribution, abundance and seasonal activity of the coneworm species. This information can be used to assist in pest management and research work on coneworm biology.

Moths of the Genus *Dioryctria* attack and kill cones of conifers throughout the Northern Hemisphere. Commonly called coneworms, the adults are small, gray or brown-orange moths with crossbands on the forewings. Four sympatric species infest pines in the South: the southern pine coneworm, *D. amatella* (Huslt); the blister coneworm, *D. clarioralis* (Walker); the webbing coneworm, *D. disclusa* Heinrich; and the loblolly pine coneworm, *D. merkei* Mutuura and Monroe. Coneworm larvae feed in the conelets, cones and stems of pines. Infested conelets and cones are destroyed making coneworms major pests in seed orchards (Ebel et al. 1980).

Mating behavior in *Dioryctria* species, as in most lepidopterans, is based on the release of a pheromone by the female. This pheromone is a volatile chemical that is very attractive to male moths. The male can locate the female by following the pheromone plume which she produces; enhancing the chances for successful mating (DeBarr and Berisford 1981).

Damage to pine seed production by coneworms has long been known (Ebel 1965, Neunzig et al. 1964, Sartor and Neel 1971). As the seed orchards established in the 1960's began to come into full production, the effects of coneworm damage became increasingly important.

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However, the trigger for a serious survey was the severe outbreak of *D. disclusa* in the late 1970's and early 1980's. Additionally, this outbreak coincided with the development of the synthetic pheromone for the webbing coneworm. Presence of a pheromone in *D. disclusa* was confirmed in 1981 (DeBarr and Berisford 1981); and the compound was soon isolated and identified as (Z)-9-tetradecenyl acetate (Meyer et al. 1982). The isolation and synthetic production of the pheromone were the essential steps for survey feasibility. The survey began in 1981, the third year of the outbreak, when USDA Forest Service personnel held training sessions, loaded pheromone baits and distributed pheromone traps to orchards throughout the South (DeBarr 1991, DeBarr et al. 1982).

Subsequent work and trapping revealed that the synthetic pheromone was attractive, not only to *D. disclusa*, but also to *D. clarioralis* and *D. merkei* (Hanula et al. 1984, Meyer et al. 1984). The final piece of the puzzle was put into place with the development of the synthetic pheromone complex for *D. amatella* (Meyer et al. 1986). Since that time, the survey has collected information on all four species.

This paper summarizes the status of the survey. Additionally, possibilities for future development of the survey are discussed.

METHODS AND PROCEDURES

Since 1982, the survey has been a cooperative effort of SEFES; FPM; and cooperating industry, state and federal orchards. The pheromone baits, traps and specific trapping procedures are described in Weatherby et al. (1985). Each year FPM sends out a request for participation to orchards in the South; in the meantime, SEFES loads the rubber septa baits with the pheromone and forwards them to FPM. After the cooperators respond, FPM, in turn, sends baits, data sheets and instructions. The cooperators are responsible for obtaining, installing and checking the traps. Cooperators check the traps twice weekly from March through November. The orchard managers can base their pest management decisions on the trap counts; however, they are also requested to forward the completed data sheets to FPM where the data are entered into a computer database (Weatherby et al. 1985).

RESULTS AND DISCUSSION

There is now a significant database on the abundance, phenology and geographical distribution of the four species of *Dioryctria* in the South. The database spans the 12 years from 1981 to 1993. Since 1985 the number of participating orchards has been, respectively: 1985 - 49, 1986 - 53, 1987 - 49, 1988 - 45, 1989 - 33, 1990 - 33, 1991 - 28, 1992 - 17. The number of orchards trapped is usually higher than the number of participants because several of the cooperators trap more than one orchard. For example, in 1993 there are 21 participants with 30 orchards being trapped. All states in the South have been represented at some time since the survey started. The cumulative numbers of participants from each state are as follows, respectively: AL - 13, AR - 4, FL - 11, GA - 11, LA - 9, MD - 1, MS - 6, NC - 10, OK - 1, SC - 7, TN - 2, TX - 5, VA - 3. Loblolly pine sources are by far the most frequently surveyed; however, slash, shortleaf, longleaf and sand pine sources have also been surveyed.

As with any cooperative project, there are some problems. It is difficult, due to personnel limitations, to provide timely feedback to participants. Additionally, maintaining a consistent database has been difficult because cooperators may not participate each year or may not trap equal durations from year to year. Participation does cost the cooperator; consequently, the yearly survey must compete with other management items that change from year to year. However, these difficulties and the usual minor coordination and communication problems have not prevented the loyal participation of several cooperators for a number of years. This has resulted in much useful data.

Data from the survey have verified and refined knowledge of the distribution and phenology of the four coneworm species. This information has been made widely available in publications such as Ebel et al. (1980). For example, *D. amatella* has several generations per year with much overlapping of life stages and is present from late April through November throughout the South. *D. clarioralis*, has three distinct generations per year in the Middle to Deep South (Ebel et al. 1980).

Much more can be done. The survey data will be useful in refinement of degree-day models that have been developed by SEFES (DeBarr, unpublished). The degree-day models will enable managers to optimally time control operations. Statistical and mathematical analyses of the data collected will help determine those factors responsible for population fluctuations of the species. Additional analyses will assess the effect of control efforts. Many of the orchards surveyed conduct routine control programs. These orchards can be compared to those where little or no control is done and the long-term effects of control measures can be assessed.

Several improvements to the survey are planned. Most immediately, the database is to be moved into a new software program. This will ease data entry and manipulation and hopefully enhance timely summaries back to cooperators.

A major change will come in the future when the survey is incorporated with the RAIN (Remote Automated Intelligence Network) computer network. RAIN consists of stations at various locations. At each location, portable computers equipped with sensors record meteorological information and automatically send the information to the central computer where it is incorporated in a database. In turn, the central computer provides this and other databases to users. Besides databases, users can access models and site-specific pest control recommendations. For example, RAIN can automatically advise managers when to apply control sprays for the Nantucket pine tip moth. Direct communication from station to station in the network is possible (Pickering et al. 1990).

Incorporation of the Southwide Coneworm Survey into the RAIN network will have several advantages. There will be more flexibility; direct communication among participants and coordinators will be possible. Cooperators will be able to directly enter their data into the database. This will eliminate the need for sending forms through the mail. Automated database updates and summaries will be possible. Consequently, responses to cooperators will be immediate, overcoming one of the most vexing problems with the present survey. RAIN will allow the correlation of trap catch data with meteorological data and degree-day models. This

will enable automated recommendations for timing of control measures. Ultimately, an expert system can be developed which uses the RAIN system to combine the survey, models, data analysis and communications software to provide orchard managers with a valuable tool to assist in management decisions.

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DESIGNING A HIGH-TECH ROOTED CUTTINGS RESEARCH FACILITY

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Abstract.--Many environmental factors are involved in the rooting of cuttings of the southern pines. Most environmental systems presently available operate such that cycles are either longer or shorter than required and rarely operate at optimum levels.

A computer automated system was designed to monitor and control cooling, heating, humidity and watering functions, all of which are top priority for rooting cuttings of the southern pines. Any of these parameters can be altered or modified through the computer, or manually controlled by override switches. The current environmental system design gives almost infinite possibilities for expanding and adding functions.

The computer is in constant two-way communication with sensors and will display the status of any area of the individual rooting benches. It also collects and logs environmental data, and accurately records all readings in each chamber, while continually monitoring for problems. This system has the capacity to keep track of and correlate all relative experimental data during the rooting period.

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QTL MAPPING IN *EUCALYPTUS* USING PSEUDO-TESTCROSS RAPD MAPS, HALF AND FULL-SIB FAMILIES.

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Key Words: RAPD, linkage maps, pseudo-testcross mapping, QTL's, *Eucalyptus*

ABSTRACT

The genus *Eucalyptus* includes the most widely used tree species for plantation establishment in tropical and subtropical regions of the world. Associations between molecular markers and quantitative trait loci (QTL's) could become a powerful tool in tree breeding to shorten breeding cycles, guide recombination and selection procedures. Particularly in *Eucalyptus*, linkage disequilibrium generated by hybridization coupled to high selection intensities and clonal propagation are conditions that would greatly favour the use of such strategies.

A pseudo-testcross mapping strategy was applied in conjunction with the high polymorphism detecting power of the RAPD (Random Amplified Polymorphic DNA) assay to construct linkage maps for single trees of two *Eucalyptus* species. At a LOD threshold of 5.0 the two maps have 242 markers on 14 linkage groups and 259 markers on 11 linkage groups respectively for *E. grandis* clone 44 and *E. urophylla* clone 28 ($n=11$ in *Eucalyptus*). A subset of evenly spaced markers that could be ordered at a 1000:1 odds established a framework map covering an estimated 96% of the genome at an average density of 11 cM.

One hundred and twenty individuals of the full-sib family used for map construction were clonally propagated and planted in a replicated trial in two locations. Height growth at 6 months and "in vitro" micropropagation ability will be evaluated in this population. A maternal interspecific half-sib family of the mapped *E. grandis* clone was analyzed at rotation age (6.5 years) in two locations in Brazil. The genetic architectures of traits with varying heritabilities are under investigation. These are wood specific gravity, volume growth and cellulose yield displaying high (0.9), medium (0.7) and low (0.4) broad sense heritabilities respectively. Simulations were used to determine statistical power for QTL detection under varying heritabilities, magnitude of QTL effect and sample sizes. A total of 1000 trees were measured for volume growth and harvested in each location. Disks at the DBH were taken on 400 trees for wood specific gravity and cellulose yield determination. Marker/QTL search is being carried out by a sequential approach including multiple bulks segregant analysis, selective individual genotyping and co-segregation analysis.

This within-half-sib approach for QTL mapping using diploid individuals is analogous to the analysis of haploid megagametophytes in conifers. Half-sibs are screened/genotyped for evenly spaced framework markers heterozygous on the maternal map. However, in the pedigree under study this approach is made even more powerful since our results show that the maternal markers are absent in the paternal pollen pool due to the genetic divergence of the two species. The pseudo-testcross mapping strategy and the approach to QTL search described should be widely applicable to any hardwood species with sufficient genetic heterogeneity. This project aims to test the feasibility of dissecting commercially important quantitative traits in fast growing *Eucalyptus* and determining the potential utility of molecular markers as a breeding tool for forest trees.

MASS VEGETATIVE PROPAGATION OF SLASH X CARIBBEAN PINE: OPERATIONAL STATUS AND RESEARCH DIRECTIONS

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Techniques developed for the rooting of juvenile cuttings of this hybrid were used to produce over one million rooted cuttings, representing six polycrossed families, for the 1993 planting season. 80% of these were raised as bare-rooted stock, the remainder as container stock for summer planting.

Shoots are harvested from an area comprising 75000 hedged stool plants. A stool plant life of three years (from seed) is used, and a third of the plants are replaced each year. When maximum production is reached in mid 1993, this shoot production area will be capable of producing annually at least four crops, each of up to one million shoots. Stool plants are established, at 40 X 40 cm spacing, in 100 X 1.1 m nursery beds covered with weed matting. A rear mounted rotary slasher with sharpened blades is used to top the stool beds at a height of 15 to 20 cm after the collection of a shoot crop has been completed.

Shoots with diameters greater than 2 mm, and primary needle lengths of over 15 mm, are cut from the stool plants to a length of 10 cm and set to a depth of 4 cm in the nursery bed. "Nursery buggies" have been developed to improve worker comfort and productivity in the collection and setting procedures. Following setting, the cuttings are covered with 50% knitted shade cloth and given 8 to 10 waterings a day each of up to seven minutes duration (applied through the shade cloth). Very effective over-the-top (and through the shade cloth) chemical weed control has been achieved. After 12 to 16 weeks, most of the cuttings have rooted, the shade cloth is removed, weekly applications of foliar fertiliser commence, and the watering is gradually reduced down to 2 or 3 ten minute waterings per day. Cuttings are conditioned for planting using undercutting, lateral pruning and topping techniques that have previously been refined for use with seedlings. Cuttings grown in the nursery bed are normally fully conditioned and ready for planting out 7 to 8 months after setting.

Container cuttings are set into locally developed 170 cc polythene tubes containing equal parts peat moss and sand, with Osmocote 8-9 Month slow release fertiliser added. Set to a depth of 2.5 cm, the cuttings are covered with 50% shade cloth and irrigated during daylight hours for one minute every 30 to 45 minutes. After 14 to 16 weeks, the shade cloth is removed and the watering is cut back to 2 or 3 waterings a day. Container cuttings are ready for planting out 6 to 7 months after setting.

All families have responded well to propagation by cuttings, with an average strike rate of 89% and utilisation rates (the percentage of shoots set that are plantable) ranging from 74% for cuttings raised in the nursery bed to 86% for container grown cuttings. In field trials, growth rate and form of cuttings are similar to those of seedlings. In an experiment subjected to cyclonic winds, cuttings of Caribbean pine displayed significantly greater windfirmness than seedlings of the same families.

Two major research directions have been followed in relation to clonal forestry. Through the application of decapitation and heavy pruning treatments, shoots with apparently juvenile rooting and growth responses can be induced to develop on trees of age 4-5 years (at which some selection is possible), but not on trees at the usual selection age of 7-8 years. The alternative approach followed has been concurrent maintenance of juvenility and clonal testing. Clonal tests involving over 250 clones will be 7 years old in 1993. The ortets of these clones were maintained in a hedged stool plant area. A preliminary assessment of these clonal tests was conducted last year, and multiplication of the more promising clones commenced. Juvenile rooting levels are evident in this material, and several of the best clones are now represented by over 1000 hedged ramets. The commercial viability of both approaches to clonal forestry is now being tested in research trials and development scale projects.

LINKAGE MAPPING USING OPEN-POLLINATED POPULATIONS

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Genomic mapping in forest trees is making fast progress. Several approaches have been employed for construction linkage maps for trees based on special features of the different species. Haploid megagametophyte tissue in the seeds, which is genetically identical with the maternal gamete, has been used to map individual mother tree for several conifer species. A pseudo-testcross strategy has been used to construct linkage maps using diploid tissues of full-sib population for *Eucalyptus*. Another approach to genomic mapping in trees has been to find appropriate 3-generation outbred pedigrees, where the 4 grandparents are available and the two parents can be crossed.

Here, we propose to construct linkage map for individual mother trees from open-pollinated population. General likelihood equation for this type of mating, which is a function of recombination frequency, allele frequency in pollen pool, and outcrossing rate, is presented in this poster. If mother genotype is known, two approaches can be used to solve the likelihood equation and get recombination estimation. The first is to put the estimated allele frequencies in pollen pool and outcrossing rate into the likelihood function before estimating the recombination frequency using a standard maximum likelihood method. The second is to estimate recombination frequency, allele frequency in the pollen pool, and outcrossing rate simultaneously using EM algorithm. If genotype of the mother tree is unknown, then EM algorithm is used to solve the likelihood equation. The power of detecting linkage, the quality of recombination frequency estimation, and the feasibility are compared with each other and are compared with a check situation where the maternal genotype, allele frequency in the pollen pool, and outcrossing rate are known.

Success of this research has at least the following impacts on genome mapping in forest trees: (1) Wide use of breeding populations will be feasible for genome mapping. In turn, genome map information can be used in breeding directly. (2) The approach proposed here, haploid megagametophyte method, and the pseudo-testcross strategy can be validated by crossing compression. (3) Mapping populations with control pollination, such as F₂, backcross, recombined inbred line etc., are actually special cases of open-pollinated populations, with different rates of outcrossing and allele frequencies in the pollen pool. This will change the design of the computer package for linkage map construction. A single unique likelihood function for open-pollinated population with specifications of outcrossing rate and the allele frequencies can be used for any type of mating. This will make the software and the algorithm generalizable.

Tree Pro Tree Shelters

James L. McConnell
U.S. Forest Service-Retired

Tree shelters or tree protectors are a new tool for planted or young trees. Current research shows that they increase growth and survival and promote straightness in many tree species. Research reported in the Journal of Forestry shows that tree protectors can increase tree height over 600 percent in just the first two years. They act as a mini-greenhouse around the tree. Research reports that planting survival is also increased in many cases.

Tree Pro is a brand name and have many advantages over other types of tree shelters.

Tree Pro Tree Protectors are:

- *Easier to install
- *Can be applied to older trees
- *Are easier to handle and transport
- *Can be left on or taken off, as necessary
- *Are made in the USA
- *Are generally cheaper than other brands and types

AN AUTOMATED APPROACH TO GENETIC MAPPING
WITH RANDOM AMPLIFIED POLYMORPHIC DNA MARKERS

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Abstract.-- At least 10000 Random Amplified Polymorphic DNA (RAPD) reactions are required to complete a modest RAPD-based genetic mapping effort. Most molecular genetics laboratories are not equipped to handle this level of production in a time-efficient manner; and efforts to scale up are costly when considering equipment acquisition and personnel needs. We describe a RAPD laboratory of our own design that is capable of completing 2304 RAPD reactions per 2.0 technician-work-days and minimal space requirements (approximately 500 square feet). The laboratory features a robotic pipettor, custom designed electrophoresis rigs, and a customized database management system. Exhibits of several recently completed RAPD-based maps are included, along with estimates of the times required to complete each map. Future plans for enhancing the throughput of the laboratory are also briefly described.

Keywords: linkage mapping, genetic markers, Random Amplified Polymorphic DNA (RAPD), Polymerase Chain Reaction (PCR), automation.

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**GENETIC MAPPING AND F2 PROGENIES RAPD ANALYSIS OF QTL'S FOR HEIGHT
GROWTH COMPONENTS OF MARITIME PINE (*Pinus pinaster* Ait.).**

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Early evaluation constitutes a challenge for forest tree breeding, but until now, only a few cases were successful (fungus or frost resistance). Early selection for height growth is much less conclusive.

One possible cause to the lack of juvenile-mature correlation could be a change in the genetic control of the traits with time. Results have been published showing differential expression of genes during maturation. Therefore a way to early predict growth performances might be to accelerate ontogenetical ageing, so as to assess adult characters on young seedlings.

We plan :

- to construct a genetic map with RAPD and Protein markers based on segregation of markers in the haploid megagametophytes of seeds of one selected inter-racial hybrid (Landes x Corsican) ;

- and then to map quantitative trait loci (QTL) influencing height growth : this cross was selected because we expect it, to be segregating for loci which determine growth components.

About 400 F2 seedlings (resulting of self-pollination of the hybrid) are growing in southwest of France under two conditions (200 each) : nursery (NC = normal condition) and accelerated condition (AC = high nutrient and continuous light) giving accelerated ageing. Around 20 traits will be measured during the first and the second growth period.

The main purposes is to compare which chromosomal segments can account for a large proportion of the genetic variation of expression of the height growth components in the two environmental conditions (NC and AC).

PLOIDY VARIATION IN EMBRYOGENIC YELLOW-POPLAR

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Tissue culture methods have been used to proliferate many plant taxa with great rapidity and in high numbers. These features alone make such methods attractive for plants having long generation times and/or low seed yields. In addition, for many agronomically important plants, genotypic variation is problematic. Somatic embryogenesis, a specific form of tissue culture technique, may potentially avoid the genotypic variation that exists in seed progeny. Rather than resulting from the fusion of meiotically "mixed" gametes in many independent fertilization events as in sexual reproduction, somatic embryos may be derived from cells of a non-sexual vegetative tissue, presumably all cells having identical genotypes. These progeny may then be considered clones of the "parent" tissue source. Field trials have shown somatic embryo-derived plants to perform comparably to seed derived-plants during the juvenile stages observed so far. We have developed and maintained somatic embryogenic yellow-poplar (*Liriodendron tulipifera* L.) tissue culture lines for several years. These cultures are maintained in a proliferative state as proembryogenic masses (PEMs), by the inclusion of plant growth regulators (2 mg/l 2,4-D, 0.5 mg/l 6-BAP) in the growth medium. Transfer of PEMs into medium devoid of growth regulators allows for the development of embryos. However, as time in culture increases, the embryogenic capacity of the tissues continues to diminish. One line maintained in culture for five years appears healthy and highly proliferative, but upon transfer to permissive medium PEMs produce embryos with very low frequency. The genetic regulation of the embryogenesis process is apparently being lost in culture over time. This breakdown is probably highly variable within a given culture, and may simply be less severe in younger aged culture lines. We have examined culture lines for cytogenetic variation using chromosomal staining techniques. Ploidy variation has been observed within culture lines. These variations in chromosomal set number may be correlated with genotype of the source tissue and time in culture. These results may demonstrate one source of variation inherent in tissue culture systems, and may also have consequences for long term tissue culture programs.

ABSTRACT

ZAMUDIO, FRANCISCO. *Age-related Variation in Growth Characteristics for Families of Pinus tecunumanii in South America.* (Under the direction of Dr. Gene Namkoong)

This paper reports the results of a planting site and age related analysis of total height (HT) and diameter (DBH) growth in fifteen half-sibs families of Pinus tecunumanii (Prov. Mt. Pine Ridge, Belize). Families were established in four field-tests by CAMCORE's organizations in South America, in 1982. Each family was planted at 3 m x 3 m spacing in a randomized complete block design, replicated nine times using six-tree row plots, and assessed for growth traits at ages three, five, and eight since planting.

Families combined large family variation with a large range of heritabilities across locations and ages, which indicate that a moderate to strong genetic control of DBH and HT exists in certain environments. Heritabilities changed with age without a clear trend related to environmental conditions.

The study of growth increments for both traits suggests that they were under a lower genetic control than the cumulative growth, and that families were less interactive with the environment after age five. Age-to-age correlations showed a highly correlated response in cumulative growth for HT and DBH, due mainly to the effects of the family variability during the first growth period. The high proportion of non-significant age-to-age phenotypic correlations for growth increments could be the effect of family variation in response to environmental fluctuations. Since total growth for diameter and height is a function of successive growth increments, and because later growth increments were poorly correlated with earlier growth, the age-to-age phenotypic correlation is misleading in most locations.

The decreasing family stability over time suggests the presence of a family x age interaction, which has to be verified in future research. Not only did families change their performance across sites at each age, but they also changed their relative performance over time.

APPENDIX

SOUTHERN FOREST TREE IMPROVEMENT COMMITTEE

Membership List

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